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14. ABSTRACT

Paget's disease (PD) of bone occurs in 3-4% of population over the age of 50. We have identified expression of measles virus nucleocapsid transcripts in osteoclast (OCL) precursors and that MVNP expression induces pagetic phenotype in osteoclasts with increased bone resorption activity as seen in patients with Paget's disease. We previously cloned and identified osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption. We hypothesize that MVNP expression in osteoclast precursors modulates RANK receptor signaling leading to Pagetic OCL development. OIP-1 blocks these signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity. We demonstrated that MVNP increases TNF-alpha induced OCL differentiation and activation by increasing NF-kB signaling through increased expression of p62, and IKK-gama and increased MAPK signaling. Our results also suggest that MVNP's effects on TNF-alpha signaling contribute to the increased OCL formation in PD. Furthermore, expression of MVNP gene in OCL in vivo induces a pagetic-like phenotype. RANKL stimulation of OIP-1 mice derived bone marrow cells resulted in significantly decreased osteoclast formation. Furthermore, OIP-1 transgenic mouse bones demonstrated an osteopetrotic phenotype. These data suggest that OIP-1 is an important physiologic regulator of osteoclast development and bone resorption in vivo and may have therapeutic utility to control excess bone turnover in patients with Paget's disease.

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Table of Contents

INTRODUCTION:

Paget's disease of bone represent the second most common bone disease after osteoporosis and affects approximately 2-3 million people in the United States. We shown that bone marrow cells from patients with Paget's disease express measles virus nucleocapsid protein (MVNP) transcripts and further demonstrated that expression of the Edmonston MVNP gene in normal osteoclast (OCL) precursors results in formation of OCL that share many of the characteristics of OCL from Paget's patients. The MVNP gene contained several sense mutations, which constituted 1% of the nucleotide sequence. The pathologic significance of MVNP and associated mutations to induce abnormal OCL formation and activity in Paget's disease, is unknown (1). RANKL is a member of Tumor necrosis factor (TNF) family member that is expressed on stromal/osteoblast cells and RANK receptor is expressed on committed osteoclast precursor cells. RANKL/RANK signaling is critical for osteoclast differentiation and bone resorption activity in vitro and in vivo (2,3). We have recently cloned and identified the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption activity. We have further demonstrated that OIP-1 significantly inhibits TNF receptor associated factor-2 (TRAF-2) and c-Jun kinase activity in osteoclast precursor cells (4). Our hypothesis is that MVNP expression in osteoclast precursors modulates the status of RANK receptor signaling molecules leading to Pagetic OCL development in Paget's disease. OIP-1 blocks these signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity in patients with Paget's disease.

BODY:

The progress on Task-1 (1-24 months) in the statement of work is as follow:

Task 1. Determine the sensitivity of MVNP transduced osteoclast precursors to RANK Ligand (RANKL) and TNF-alpha stimulation to form pagetic osteoclasts (Months 1-24):

(a) **Test the dose response effect of RANKL and TNF-alpha on MVNP transduced osteoclast precursors to form Pagetic osteoclasts (Months 1-7)**.— We have developed retroviruses to transduce osteoclast precursors with empty vector or MVNP to assess the effects on osteoclasts. We have tested the dose response effect of RANK ligand (RANKL) on MVNP induced osteoclastogenesis. Bone marrow derived osteoclast precursor cells (CFU-GM) transduced with MVNP were stimulated with various concentrations of RANKL in the presence of M-CSF (10 ng/ml) for 7 days. Empty vector transduced cells served as control to these experiments. As shown in Fig.1a, RANKL enhanced MVNP stimulation of osteoclastogenesis in a dose dependent manner. MVNP transduced osteoclast precursosrs resulted in formation of pagetic osteoclasts (Fig.1B) as identified by tartrate resistant acid phosphatase (TRAP) activity staining. The nuclear number in the MVNP transduced osteoclasts were

increased significantly. Similarly, MVNP transduced normal human CFU-GM showed increased responsivity to TNF-alpha approximately two-fold. Task-2 studies ongoing will further delineate associated signaling mechanisms using RAW264.7 mouse macrophage cell line which can differentiate to osteoclasts in the presence of RANKL.

Fig.1a

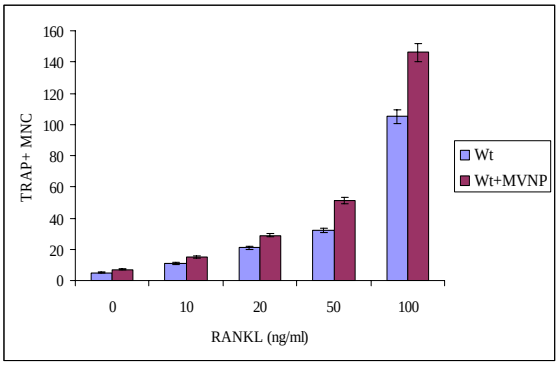


Fig.1b

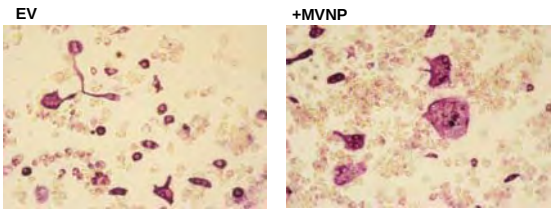


Fig.1b

Fig.1 Osteoclast formation in MVNP transduced bone marrow cells. (a) Osteoclast precursor cells were transduced with MVNP gene and cultured for osteoclast in presence of M-CSF (10ng/ml) and RANKL at varying concentration (0-100ng/ml) for 7 days. TRAP multinucleated cells were scored using inverted light microscope. The results are expressed as mean ± SD for triplicate cultures. (b) Morphology of multinucleated cells formed from TRAP+ osteoclast cultured with and without MVNP transduced gene.

(b) Assess the capacity of osteoprotegerin (OPG) to block RANKL and TNF-alpha induced osteoclast differentiation of MVNP transduced osteoclast precursors (Months 7-17). --- We examined if MVNP transduction will increase osteoclat precursor proliferation. Human bone marrow derived osteoclast precursor cells (CFU-GM) cells were cultured with and without MVNP transduction. As shown in Fig.2, MVNP transduction significantly increased osteoclast precursor growth in methyl cellulose cultures. As noted above (task1a), TNF-alpha stimulation further enhanced by a two-fold increase in osteoclast precursor growth in MVNP transduced cells. We did not detect a significant effect of OPG on MVNP or TNF stimulated osteoclast precursor growth or osteoclast development in MVNP enhanced osteoclast formation.

Fig.2

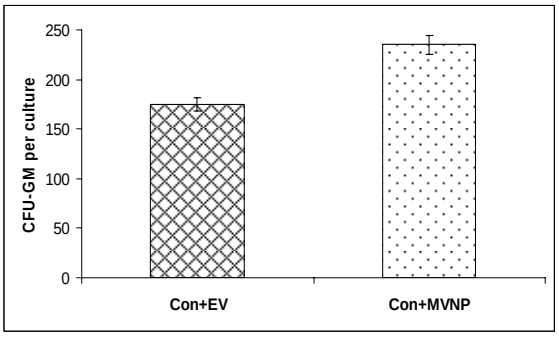


Fig.2. CFU-GM colony formation in MVNP transfuced human bone marrow. Human bone marrow cells (4 $\times 10^5$/ml) were cultured with hGM-CSF (10ng/ml) in methyl cellulose (10%) to form CFU-GM colonies with and without MVNP transduction. At the end of 7 days culture period, CFU-GM colonies formed in these cultures were scored using a light microscope ($P<0.05$).

(c) Determine the effects of anti-IL-6 and IL-6 on the responsivity of MVNP transduced osteoclast precursors to RANKL and TNF-alpha stimulation (Months 17-24).— To address this task, we examined if MVNP transduction will increase IL-6 production by the osteoclast precursor cells. Osteoclast precursor cells transduced with MVNP or empty vector were cultured for 7 days in the presence and absence of RANKL. As shown in Fig.3a, we observe a significant increase in the levels of IL-6 in the conditioned media obtained from MVNP transduced cultures in the presence of RANKL. We did not see significant effect of TNF-alpha to enhance IL-6 levels in MVNP transduced osteoclast precursor cells in the presence or absence of RANKL. We further examined if a neutralizing antibody against IL-6 can block MVNP enhanced osteoclasat formation. As shown in Fig.3b, antibody against IL-6 significantly decreased MVNP stimulated osteoclast formation. In contrast IL-6 antibody has no significant effect in control cultures transduced with an empty vector. A non-spsecific control IgG also has no significant effect on MVNP enhanced osteoclast formation. These results suggest that MVNP transduction will increase IL-6 production significantly and that IL-6 may play an important role in MVNP stimulated osteoclast formation.

Fig. 3a

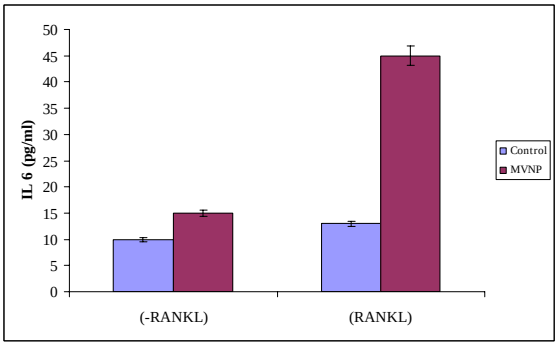
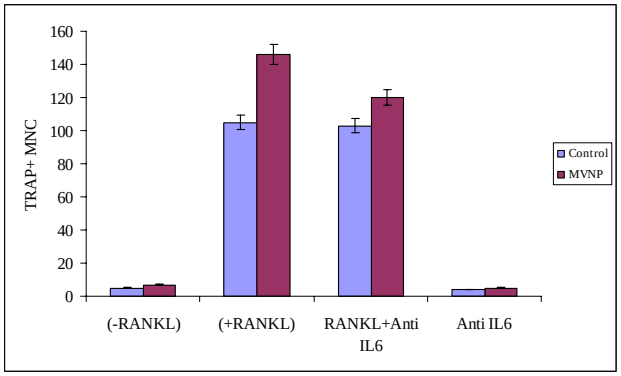


Fig.3 (A) IL-6 production. Mouse bone marrow derived osteoclast precursor cells were transduced with MVNP gene with or without RANKL (100ng/ml) for 7 days. Conditioned media were harvested at 7 days, and the concentration of IL-6 was determined using ELISA. Results are reported as IL-6 concentration (pg/ml) and are the mean ± SD of triplicate samples.

Fig.3b.



3b. Effects of anti-IL-6 on bone marrow cultures on MVNP transduction. Bone marrow cells (10^5 cells/well) were transduced with MVNP and culture with and without RANKL (100ng/ml) and in the presence or absence of 50 ng/ml anti-mouse IL-6 or non-specific control IgG. After 7 days of culture, the cells were fixed with 2% glutaraldehyde and stained for TRAP activity. The $TRAP^+$ multinucleated cells were scored using an inverted microscope. The results are expressed as mean ± SE for triplicate cultures.

Task 2. Determine the RANK receptor signaling in MVNP transduced osteoclast precursors (Months 17-36)—**Not yet initiated.**

Task 3. Determine the effects of OIP-1 on MVNP altered RANK receptor signaling in osteoclast precursor cells (Months 29-48). ---**Not yet initiated**.

KEY RESEARCH ACCOMPLISHMENTS:

- We have developed MVNP retroviruses expression constructs for transduction into osteoclast precursor cells.
- We have identified MVNP transduction will enhance IL-6 production by the osteoclast precursor cells.
- We have shown anti-IL-6 significantly decrease MVNP stimulated osteoclastogenesis.

REPORTABLE OUTCOMES:

Articles:

1. **Reddy SV**. Etiology of Paget's disease and osteoclast abnormalities. J. Cellular Biochem., 93:688-696, 2004.
2. **Reddy SV**. Regulatory mechanisms operative in osteoclasts, Crit. Rev. Eukaryot. Gene Expr. 14:255-270, 2004.

Abstracts:

1. G. D. Roodman, **S. V. Reddy**, N. Kurihara, J. J. Windle, L. A. Ehrlich, H. Zhou, D. Dempster, M. Subler. Measles Virus Nucleocapsid (MVNP) Gene Is Sufficient To Induce a Pagetic Bone Phenotype in Vivo. ASBMR Oct. 2004, Seattle

2. M. Ito, H. Kajiya, N. Kawanabe, N. Kurihara, T. L. Johnson-Pais, J. J. Windle, **S. V. Reddy**. Further Characterization of Human Osteoclast Inhibitory Peptide-1 (OIP-1/hSca) Gene Expression. ASBMR Oct. 2004, Seattle

3. M. Ito, N. Kurihara, **S. V. Reddy**, G. D. Roodman. Measles Virus Nucleocapsid Protein (MVNP) Increases p62 and IKK-gamma Expression to Enhance TNF-alpha-induced Osteoclast (OCL) Differentiation and Activation in Paget's disease. ASBMR Oct. 2004, Seattle

CONCLUSIONS:

In conclusion, our results demonstrate that MVNP increases transduction to osteoclst precursor cells will enhance IL-6 production and stimulate osteoclast formation. Antibody against IL-6 significantly neutralizes MVNP stimulated osteoclast formation. Also, TNF-alpha further enhances MVNP stimulated osteoclast formation. However OPG has no significant on MVNP or TNF-alpha stimulated osteoclast formation. These results implicate a potential role for IL-6 in MVNP enhanced osteoclastogenesis in pathogenesis of paget's disease of bone.

REFERENCES:

1. S.L. Teitelbaum, F.P. Ross, *Nat. Rev. Genet.* **4,** 638 (2003)

2. G. D. Roodman, Windle JJ, *J Clin Invest.* **115,** 200 (2005)

3. J.L. Roccisana *et al., J. Biol. Chem.* **279,** 10500 (2004).

4. M. Koide, H. Maeda *et al., J Bone Miner Res.* **18,** 458 (2003).

APPENDICES:

Reprints enclosed for two relevant articles as noted under outcomes.

Journal of Cellular Biochemistry 93:688–696 (2004)

Etiology of Paget's Disease and Osteoclast Abnormalities

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Abstract Paget's disease of bone is a chronic focal skeletal disorder that affects up to 2–3% of the population over the age of 60 years. Paget's disease is primarily a disease of the osteoclast. The pathologic abnormality in patients with Paget's disease involves increased bone resorption by the osteoclasts, followed by abundant new bone formation that is of poor quality. Genetic linkage analysis indicated that 40% of patients with Paget's disease have an affected first degree relative and 1% of patients develop osteosarcoma. Paget's disease is an autosomal dominant trait with genetic heterogeneity. Recurrent mutations in the ubiquitin-associated (UBA) domain of sequestosome 1 (SQSTM1/p62) are identified in patients with Paget's disease. Osteoclasts and osteoclast precursors from patients with Paget's disease contain paramyxoviral transcripts and appear hyperresponsive to 1,25-$(OH)_2D_3$ and RANK ligand (RANKL). It has been suggested that the enhanced sensitivity of osteoclast precursors for 1,25-$(OH)_2D_3$ in Paget's disease results from increased expression of coactivators of vitamin D receptor (VDR). However, a cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations associated with this disease and osteoclast abnormalities are unclear. Therefore, the etiology of Paget's disease remains uncertain. J. Cell. Biochem. 93: 688–696, 2004.

Key words: Paget's disease; osteoclast; measles virus (MV); sequestosome (p62); tartrate resistant acid phosphatase (TRAP); RANK ligand (RANKL)

Sir James Paget first described Paget's disease of bone in 1877 as Osteitis Deformans, a chronic focal skeletal disease that can be monostotic or polyostotic. Paget's disease is the second most common metabolic bone disease and affects between 2% and 3% of the population over the age of 60. The disease been associated with deformity and enlargement of single or multiple bones among which, the skull, clavicles, long bones, and vertebral bodies are the most frequently involved [Paget, 1877]. Patients with Paget's disease are frequently asymptomatic, but approximately 10–15% of the patients have severe symptoms including bone pain, fractures, neurological complications due to spinal cord compression, deafness, and dental abnormalities. Paget's disease is a highly localized disease, and new lesions rarely develop during the course of the disease. Rather, lesions continue to progress in size unless treated. The primary pathologic abnormality in patients with Paget's disease is increased bone resorption, followed by abundant new bone formation. The bone that is formed is disorganized and of poor quality, resulting in bowing of the bone, stress fractures, and arthritis in joints contiguous to the involved bones. In addition, patients with Paget's disease can develop hypercalciuria and hypercalcemia, due to accelerated bone resorption induced by immobilization. Another interesting feature of Paget's disease is that bones not clinically involved with Paget's disease appear to show increased bone remodeling. This increased bone remodeling in unaffected bones has been ascribed to secondary hyperparathyroidism rather than to subclinical involvement of the bones with Paget's disease. However, less than 20% of patients with Paget's disease have elevated parathyroid hormone (PTH) levels [Siris, 1999]. A juvenile form of Paget's disease also called hyperostosis corticalis deformans juvenilis or hereditary hyperphosphatasia is very different than the adult form of

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the disease. It is characterized by widespread involvement of the skeleton and has histological and radiological features that distinguish it from Paget's disease of the adults, including the absence of viral-like nuclear inclusions in osteoclasts present in the bone microenvironment [Whyte et al., 2002].

Bone scans are the most sensitive method of detecting pagetic lesions and can be used to follow the activity of the disease in these patients. Initial lesions appear osteolytic, followed by a chaotic sclerotic appearance, and finally become osteosclerotic. Considerable thickening of the sclerotic bone results in bone deformity. Serum tartrate resistant acid phosphatase (TRAP), presumably released by osteoclasts, appears to be an index of bone resorption in Paget's disease but is not routinely used. The most useful markers for the increased osteoblast activity in Paget's disease are the total alkaline phosphatase and bone-specific alkaline phosphatase activity levels in serum. Patients also showed significantly higher endothelin-1 circulating levels than controls with a positive correlation with serum alkaline phosphatase, but not with urinary hydroxyproline [Tarquini et al., 1998]. Furthermore, serum calcium levels are typically normal in Paget's disease and also serum osteocalcin levels appear to be a poor index of the progression of the disease. Bisphosphonates are the most common treatment for patients with Paget's disease. These inorganic phosphate compounds inhibit osteoclast-mediated bone resorption and induce osteoclast apoptosis [Siris, 1999]. It has been reported that serum M-CSF levels are significantly elevated in patients with Paget's disease, however not significantly different in patients under treatment compared to normal subjects [Neale et al., 2002].

Paget's disease has a very unusual geographic distribution, with an increased incidence in Caucasians of European descent, but it also occurs in African Americans. It is rare in those of Asian descent. Studies also suggested high prevalence rates of radiographic Paget's disease in Britain, Australia, North America, and Western Europe. Interestingly, even within Britain there is a marked geographic variation in the incidence of Paget's disease, with an increased incidence in the Western portion of England and a much lower incidence in the Southern portion of England (8% vs. 4%) [Cooper et al., 1999]. The prevalence of the disease is 2.5% among men and 1.6 % among women aged 55 years and above in British towns. The level of prevalence in Spain is estimated to be at 1.5%. A radiographic survey of 24 patients with Paget's disease in Ireland further revealed monostotic disease in 8 and polyostotic disease in 16. There have been reports that one to three million patients over the age of 55 are affected with Paget's disease in the United States. This is in contrast with the extreme rarity of the disease in Scandinavia, Ireland, and Southern Europe. This unusual geographic distribution for the incidence of Paget's disease is not attributable to geographic, environmental, or industrial exposures in these areas and currently cannot be explained. Furthermore, the incidence of Paget's disease appears to be decreasing over the last several decades [Siris, 1999; Doyle et al., 2002], but the basis for this decrease in the incidence of Paget's disease is unknown. However, it is evident that genetic factors play important role in the familial and sporadic forms of Paget's disease. Furthermore, Paget's disease has been described as a slow paramyxoviral infection process, suggesting a viral etiology for the disease. Therefore, this review will focus on the etiology of Paget's disease with an emphasis on the role that genetic and paramyxoviral infection may play in abnormal osteoclast development responsible for excess bone resorption in patients with Paget's disease.

GENETICS OF PAGET'S DISEASE

Familial incidence is common in Paget's disease and 40% of patients with the disease have an affected first-degree relative. Therefore, genetic factors play an important role in the pathogenesis of Paget's disease of bone. The disease often is inherited in an autosomal dominant manner manifesting genetic heterogeneity and incomplete penetrance. Familial Paget's disease has an equal incidence in males and females. A genetic locus for Paget's disease has been identified on chromosome 18q [Leach et al., 2001; Good et al., 2002] in several large families with Paget's disease in a region near the familial expansile osteolysis (FEO) locus. FEO is a disease related to Paget's disease but occurring in patients at a much younger age and being a much more severe disease. FEO is an extremely rare disease, affecting only a very limited number of kindreds in the world, also mapped to chromosome 18q and is linked to activating mutations in the TNFRSF11A gene

which encodes receptor activator of nuclear factor κB (RANK) [Hughes et al., 2000]. Recently, in patients with Juvenile Paget's disease, a homozygous deletion of the gene on chromosome 8q24.2 that encodes osteoprotegerin, member of the superfamily of tumor necrosis factor receptors, has been reported [Whyte et al., 2002]. However, linkage studies, coupled with mutation screening have excluded involvement of RANK and also osteoprotegerin in the majority of patients with Paget's disease of bone [Sparks et al., 2001]. Studies also indicated that patients with Paget's disease have an increased incidence of osteosarcoma, with approximately 1% of patients with Paget's disease developing osteosarcoma in an affected bone. This incidence of osteosarcoma is 1,000 times higher than that in the general population for this age group. Recent genetic studies have demonstrated linkage in seven of seven patients with osteosarcoma to loss of heterozygosity in a region of 18q that is adjacent to or within a locus for Paget's disease on 18q [Hansen et al., 1999].

A genome wide search in familial Paget's disease of bone further indicated genetic heterogeneity of the disease with candidate loci on chromosomes 2q, 10q, and 5q [Hocking et al., 2001]. More recently, the gene encoding sequestosome 1 (SQSTM1/p62) mapped within the critical region on chromosome 5q35-qter identified a proline-leucine amino acid change at codon 392 (P392L) in French-Canadian patients with Paget's disease of bone [Laurin et al., 2002]. The frequency of mutation was 16% and 46% for sporadic and familial cases tested, respectively. Further studies also identified different mutations affecting the highly conserved ubiquitin-binding domain of SQSTM1/p62 protein in patients with familial and sporadic Paget's disease [Johnson-Pais et al., 2003; Good et al., 2004; Hocking et al., 2004].

ROLE OF SQSTM1/P62 IN OSTEOCLASTOGENESIS

The atypical PKC (aPKC) interaction with SQSTM1/p62 has been implicated in signaling cascades that control NF-κB activation. It is evident that p62 provides a scaffold linking the aPKCs to the tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) receptor signaling complexes through its interaction with RIP and TRAF-6, respectively [Moscat and Diaz-Meco, 2000]. Thus, SQSTM1/p62 mediate IL-1 and TNF-α cytokine signaling to activate NF-κB (Fig. 1). TRAF-6 plays an essential role in RANK ligand (RANKL) signaling during osteoclastogenesis. More recently, it has been shown that RANKL stimulation results in upregulation of p62 expression in osteoclast precursor cells and that the genetic inactivation of p62 in mice impaired PTHrP induced osteoclastogenesis in vivo. However, p62 null mice have grossly normal skeletal phenotype and that no alterations were found in the trabecular size and number of osteoclasts compared to wild-type mice. In vitro studies further demonstrated that p62 deficiency leads to inhibition of IKK activation and NF-κB nuclear translocation during osteoclastogenesis [Duran et al., 2004]. These studies also demonstrated that RANKL stimulation induces formation of a ternary complex involving TRAF-6, p62, and aPKC during osteoclastogenesis. Recent studies also identified novel mutations in ubiquitin-associated (UBA) domain of SQSTM1/p62, however, genotype-phenotype analysis indicated that there is no correlation with respect to different mutations in UBA and disease occurrence [Hocking et al., 2004]. Therefore, the precise role that SQSTM1/p62 may play in the pathogenesis of Paget's disease of bone remains to be elucidated.

VIRAL ETIOLOGY

Since the early 1970s, a variety of studies have implicated paramyxoviruses in Paget's disease. The viral etiology has been proposed for Paget's disease with an initial description of nucleocapsid-like structures in the nuclei and cytoplasm of pagetic osteoclasts by electron microscopy [Mills and Singer, 1976]. Immunocytochemical studies further confirmed that these nuclear inclusions cross-reacted with antibodies that recognized measles virus (MV) or respiratory syncytial virus (RSV) nucleocapsid antigens. In situ hybridization techniques also identified the presence of MV messenger RNA (mRNA) sequences in up to 90% of osteoclasts and other mononuclear cells in pagetic bone specimens. Similarly, canine distemper virus (CDV) nucleocapsid antigens were also detected in osteoclasts from patients with Paget's disease. These paramyxoviral-like nuclear inclusions are not unique to Paget's disease and were reported in patients with FEO and rarely in patients with osteopetrosis, pycnodysostosis, and otosclerosis, oxalosis [Singer, 1999]. This has raised the

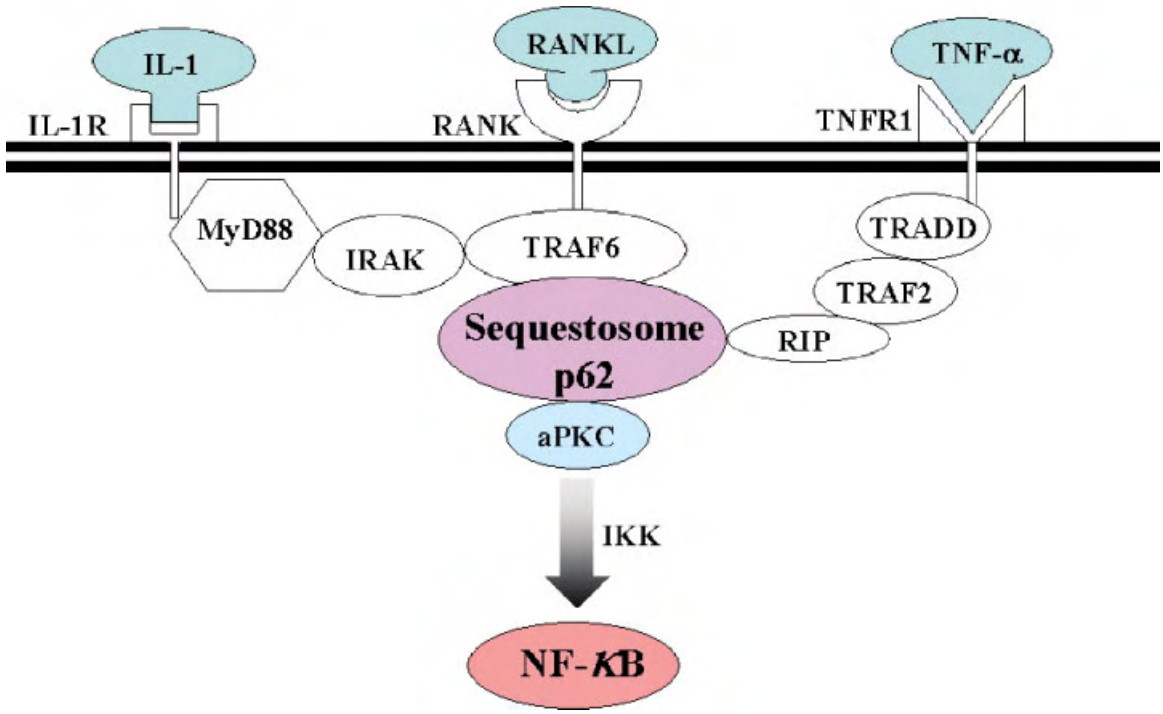

Fig. 1. Signaling cascades associated with sequestosome/p62. Sequestosome provides a scaffold linking the aPKCs to the TNF-α and IL-1 receptor signaling complexes through its interactions with RIP and TRAF-6, respectively resulting in phosphorylation of IKK and activation of NF-κB. RANKL-RANK signaling induce p62 to form a ternary complex with TRAF-6 and aPKCs during osteoclast differentiation [Duran et al., 2004]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

possibility that that the virus may be a non-etiologic agent in a cell altered by a genetic defect.

To further explore the viral etiology, using reverse transcription-polymerase chain reaction (RT-PCR) analysis, we have amplified the MV nucleocapsid (MVNP) transcripts from freshly isolated bone marrow cells from patients with Paget's disease. These MVNP transcripts contain mutations clustered at the c-terminal end of the mRNA [Reddy et al., 1995]. All these mutations were sense mutations and resulted in amino acid substitutions in the nucleocapsid gene product. The mutations occurred at 1% rate in the total MVNP gene isolated from a patient with Paget's disease. We further demonstrated that osteoclast precursors, the granulocyte macrophage colony-forming unit (CFU-GM), as well as mature osteoclasts from patients with Paget's disease, expressed MVNP transcripts. Since CFU-GM circulate and also give rise to monocytes and granulocytes in the peripheral blood, we then examined peripheral blood mononuclear cells from patients with Paget's disease and normals for expression of MVNP transcripts. We found by RT-PCR analysis that peripheral blood samples from 9 of 10 patients with Paget's disease contain MVNP transcripts, while none of the 10 normals tested expressed MVNP transcripts [Reddy et al., 2001a]. We were unable to find CDV or RSV nucleocapsid transcripts in patients we have studied. In contrast, CDV nucleocapsid transcripts were detected in affected bones from 100% of patients tested using in situ RT-PCR techniques. Furthermore, it has also been demonstrated that infecting canine bone marrow cells with CDV results in development of multinucleated cells that share some of the phenotypic characteristics of pagetic osteoclasts [Gordon et al., 1992]. However, other workers have been unable to detect paramyxoviral nucleocapsid transcripts in samples obtained from patients with Paget's disease [Helfrich et al., 2000; Ooi et al., 2000].

The presence of MV or CDV transcripts in osteoclasts and osteoclast precursors from patients with Paget's disease does not infer a

pathophysiologic role for these genes in the development of the pagetic lesions. It is possible that these paramyxoviral transcripts and paramyxoviral-like inclusions are simply markers for the disease and have no pathophysiologic significance. In studies, using normal osteoclast precursors (CFU-GM) transduced with retroviral vectors expressing the MVNP gene formed large osteoclasts more rapidly with an increased numbers of nuclei, hypersensitive to 1,25-dihydroxyvitamin D3 (1,25-$[OH]_2D_3$) and had increased bone resorbing capacity compared to normal osteoclasts. In contrast, normal osteoclast precursors transduced with the MV matrix gene did not express an abnormal phenotype [Kurihara et al., 2000]. In further studies, we have targeted CD46, human MV receptor to cells of the osteoclast lineage in transgenic mice and demonstrated that MV infection of osteoclast precursors from CD46 transgenic mice form osteoclasts, which express a pagetic phenotype in vitro [Reddy et al., 2001b]. Taken together, these data suggest a potential pathophysiologic role for the paramyxoviral nucleocapsid gene that is expressed in patients with Paget's disease. Mouse models of MV infection were also developed in which CD46 is introduced into transgenic mice and has been bred to another transgenic mouse lacking the alpha-beta-interferon receptor. Upon exposure to MV, these mice developed immune-suppression similar to patients with acute MV infection. The mice lack the alpha-beta interferon receptor demonstrated persistence of MV infection for at least 12 days [Peng et al., 2003]. However, TRAP-CD46 mice do not develop sustained MV infection, most likely reflecting the need for blocking interferon production for development of persistent MV infection in these mice.

MV infection has a similar incidence worldwide and occurs in very young patients, whereas Paget's disease is a disease of the elderly. These observations suggest that if paramyxoviruses have an etiologic role in Paget's disease, these viral infections must persist for long periods of time. To further investigate a potential site for the initial infection of osteoclast precursors with Paget's disease, we tested the hypothesis that very early pluripotent hematopoietic stem cells, which can persist for long periods of time in a quiescent phase, may be the initial target for the paramyxoviral infection in patients with Paget's disease. We found that other hematopoietic lineages from patients with Paget's disease in addition to the osteoclast lineage, including the erythroid and the erythroid precursors, burst-forming unit-erythroid (BFU-E), and multipotent myeloid precursors (CFU-GEMM), which give rise to megakaryocytes, monocytes, erythroid cells, and granulocytes, also contain paramyxoviral nucleocapsid transcripts [Reddy et al., 2001a]. Thus, if the initial site of infection occurs in a small number of primitive pluripotent hematopoietic stem cells that predominantly remain in Go, this might explain the chronicity of the infection. Furthermore, there may be a genetic predisposition for chronic paramyxoviral infections of hematopoietic precursors in patients with Paget's disease. However, a cause and effect relationship of paramyxoviruses in Paget's disease remains proven as yet no infectious virus been isolated from pagetic cells and also, it is not clear how the initial lesion occurs in Paget's disease.

PAGETIC OSTEOCLASTS

Histologic examination of pagetic bone biopsy revealed abundant structurally abnormal osteoclasts. Osteoclasts are increased in number and size, and contain as many as 100 nuclei per multinucleated cell compared to three to five nuclei for a normal osteoclast. These osteoclasts have characteristic ultrastructural abnormalities including microfilaments, paracrystalline arrays located in the nucleus and sometimes in the cytoplasm that are absent in non-pagetic bone or bone marrow cells. These inclusions closely resemble nucleocapsids of viruses of the paramyxoviridae family [Mills and Singer, 1976]. Osteoblasts are also increased in lesions in patients with Paget's disease, and they appear to be morphologically normal. Osteoblasts contain abundant rough endoplasmic reticulum and mitochondria in a well-developed Golgi zone, consistent with the increased bone formation activity that occurs in the active lesions. In advanced lesions in patients with Paget's disease, the marrow is also abnormal. The bone matrix in Paget's disease is highly abnormal in structure due to disordered bone remodeling. The bone matrix consists of erratic patterns of "cement lines" and demonstrates a "mosaic" pattern. The matrix is interspersed with numerous foci of woven bone, reflecting the increased rates of bone deposition that is of poor quality.

The bone marrow culture techniques identified several abnormalities in osteoclast formation and osteoclast precursors from patients with Paget's disease. Osteoclast-like multinucleated cells formed more rapidly with increased numbers (10–100-fold) and nuclei per osteoclast, expressed high levels of TRAP in marrow cultures from patients with Paget's disease compared to normals. In addition, osteoclast formation in pagetic bone marrow cultures was induced at concentrations of 1,25-$(OH)_2D_3$ that were 10–100 times lower than those required in normal marrow cultures. Structural examination of the osteoclast-like cells formed in bone marrow cultures also showed that they had many of the features of pagetic osteoclasts but lacked the characteristic nuclear and cytoplasmic inclusions. Immunocytochemical studies confirmed that MV and RSV nucleocapsid antigens were expressed in osteoclasts formed in vitro in these cultures. Osteoclasts from patients with Paget's disease also appear to produce increased levels of IL-6 and express high levels of IL-6 receptors compared to normal osteoclasts. In situ hybridization studies have further identified increased levels of IL-6, c-fos proto-oncogene, Bcl 2 anti-apoptotic gene mRNA expression in pagetic osteoclasts. IL-6 receptor and NF-IL-6 mRNA levels were also increased in osteoclasts from bone samples from patients with Paget's disease compared to those with osteoarthritis [Hoyland et al., 1994]. These data suggest that IL-6, which is a stimulator of human osteoclast formation, may act as an autocrine/paracrine factor to enhance osteoclast formation in patients with Paget's disease and increase the osteoclast precursor pool. IL-6 levels were also shown to increase in bone marrow plasma and peripheral blood of patients with Paget's disease [Roodman et al., 1992]. In addition, the increased levels of IL-6 in the peripheral blood of patients with Paget's disease may in part explain the increased bone remodeling seen in bones not clinically involved with Paget's disease.

To further investigate the potential abnormalities in osteoclast precursors in patients with Paget's disease, the number of osteoclast precursors in marrow aspirates from involved bones from patients with Paget's disease were assessed. It has been found that the number of early osteoclast precursors, CFU-GM, was increased significantly in marrow aspirates from patients with Paget's disease compared to normals. Interestingly, when the osteoclast precursors were separated from the marrow microenvironmental elements present in the marrow aspirates, similar numbers of osteoclast precursors were detected in these aspirates. These data suggested that the marrow microenvironment enhanced osteoclast precursor growth compared to the normal marrow microenvironment.

To determine the potential role of the marrow microenvironment and the enhanced osteoclast formation in patients with Paget's disease, reconstitution experiments were conducted using highly purified populations of osteoclast precursors from patients with Paget's disease or normals and marrow stromal cells from patients with Paget's disease and normals. Coculture of normal osteoclast precursors with marrow stromal cells from patients with Paget's disease resulted in enhanced growth of the osteoclast precursors from normals. Interestingly, when osteoclast precursors from patients with Paget's disease were cocultured with marrow stromal cells from normals, they also showed increased growth. These data suggest that both the marrow microenvironment, as well as the osteoclast precursors, are abnormal in patients with Paget's disease.

These studies also confirmed that the osteoclast precursors were hypersensitive to 1,25-$(OH)_2D_3$ compared to normals. The increased sensitivity of osteoclast precursors from Paget's patients to 1,25-$(OH)_2D_3$ is mediated through the vitamin D3 receptor (VDR). This was confirmed by upregulation of 24-hydroxylase mRNA expression in pagetic osteoclast precursors at concentrations of 1,25-$(OH)_2D_3$ that are one log less than that required for normal osteoclast precursors. The increased sensitivity to 1,25-$(OH)_2D_3$ was not due to increased numbers of vitamin D receptors in pagetic osteoclast precursors compared to normals, but appeared to be due to enhanced affinity of the VDR in pagetic cells for its ligand compared to normals [Menaa et al., 2000a]. Recently, it has been demonstrated that MVNP gene expression in osteoclast precursors results in increased levels of TAF_{II}-17 transcription factor gene expression. The high levels of TAF_{II}-17 permit formation of VDR transcription complex at low levels of receptor occupancy by 1,25-$(OH)_2D_3$ [Kurihara et al., 2004]. These results support the hypothesis that part of the pathophysiology underlying the increased osteoclast activity in Paget's

disease is due to increased levels of VDR coactivators that enhance VDR-mediated gene transcription at low levels of 1,25-$(OH)_2D_3$. These studies suggested that Paget's disease may be a VDR coactivator disease.

The osteoclast precursors from patients with Paget's disease also appear to be hyperresponsive to receptor activator of NF-κB ligand (RANKL) and that marrow stromal cells from pagetic lesions have increased RANKL expression [Neale et al., 2000; Menaa et al., 2000b]. RANKL is a critical osteoclast differentiation factor that is expressed on marrow stromal and osteoblast cells in response to several osteotropic factors. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with interleukin-6 (IL-6). Addition of neutralizing antibodies to IL-6 decreased the sensitivity of the osteoclast precursors from patients with Paget's disease to RANKL to normal levels. Similarly, addition of IL-6 to cultures of normal osteoclast precursors enhanced the responsivity of these precursors to RANKL to the levels seen with pagetic osteoclast precursors. Pagetic osteoclasts expressing MVNP gene produce high levels of cytokines that increase osteoclast precursor pool as well as osteoblast precursor proliferation and constitutive expression of RANKL, which contribute to the abnormal osteoclast development and highly localized nature of Paget's disease (Fig. 2). Immature osteoblasts are the major responders to RANKL inducing cytokines and studies also suggested that expression of RANKL decreases with osteoblast maturation [Gori et al., 2000]. Therefore, the increased numbers of highly active osteoblasts rapidly form large amounts of woven bone in patients with Paget's disease. Furthermore, the prodigious amounts of cytokine production by the pagetic osteoclasts result in continued stimulation of osteoblast precursors growth making the local microenvironment in the pagetic lesion progressively more osteoclastogenic resulting in elevated levels of bone resorption in these patients. Consistent with this hypothesis are findings of high levels of cytokines such as IL-6 being produced by pagetic osteoclasts and the increased RANKL protein levels in marrow adherent cells from pagetic lesions compared to normal marrow and uninvolved bones from the same patient [Menaa et al., 2000b]. In addition, the clinical observation that inhibiting osteoclast formation with bisphosphonates results in a dramatic fall in alkaline phosphatase in

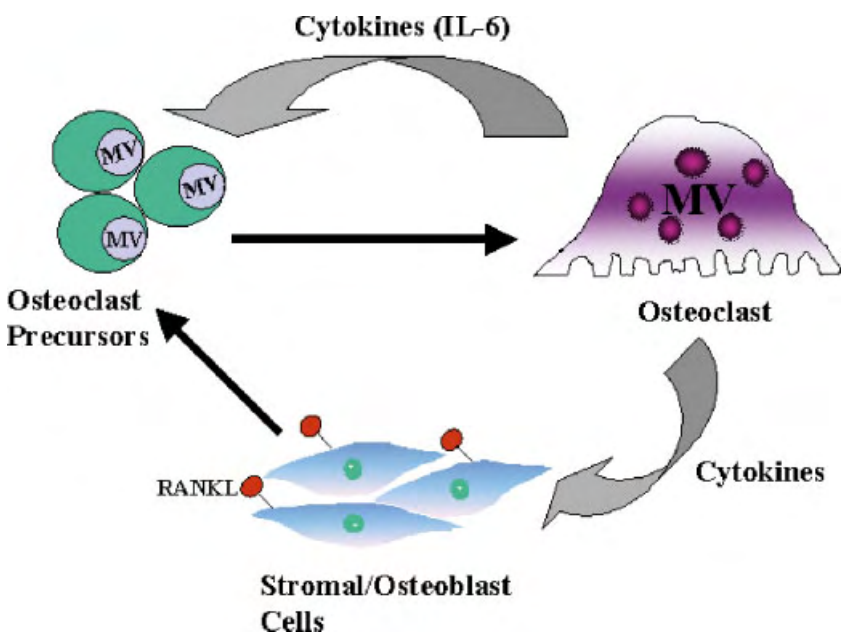

Fig. 2. Osteoclastogenesis in pagetic bone microenvironment. The osteoclast precursors contain measles virus (MV) transcripts and are hyperresponsive to RANK ligand (RANKL). The pagetic osteoclasts produce increased levels of cytokines such as IL-6, which enhance osteoclast formation. Chronic exposure to cytokines produced by the pagetic osteoclasts results in constitutive overexpression of RANKL in stromal/osteoblast cells further enhancing the abnormal osteoclast development in pagetic bone lesions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

patients with Paget's disease, demonstrates that osteoclasts are driving the osteoblastic response in Paget's disease. Alternatively, pagetic osteoclasts may live for very long periods of time compared to normal osteoclasts and persist in the lesion. It has been reported that the anti-apoptotic gene Bcl-2 is overexpressed in pagetic osteoclasts, suggesting that the osteoclasts lifespan may be prolonged in pagetic lesions. More recently, it has also been reported that mutations in the Bcl-2 gene promoter are responsible for upregulation of Bcl-2 expression leading to enhanced osteoclastogenesis in patients with Paget's disease [Brandwood et al., 2003]. Recently, it has been shown that SHIP, inositol 5′ phosphatase deficient mice are severely osteoporotic with an increased numbers of osteoclast precursors and hyperactive osteoclasts. In addition, serum levels of IL-6 are markedly increased in these mice as in Paget's disease [Takeshita et al., 2002]. However, the basis for these abnormalities in both osteoclasts and osteoclast precursors from patients with Paget's disease is still unknown.

SUMMARY AND FUTURE DIRECTIONS

Paget's disease of bone is the second most disorder of bone after osteoporosis. The disease is an autosomal dominant trait with genetic heterogeneity. The recent discovery of recurrent mutations occurring in the UBA domain of SQSTM1/p62 in patients with Paget's disease suggests that genetic factors may play an important role. Although SQSTM1/p62 mutations are implicated as a common cause of familial and sporadic Paget's disease, there is no correlation among different mutant forms of p62 and disease severity. Lack of skeletal abnormalities in p62 null mice further suggests a potential role for genes present in other candidate loci that were linked with Paget's disease. Alternatively, a genetic defect may favor the environmental factors such as MV infection to have potential role in pathogenesis of the disease. However, the molecular basis for the abnormalities associated with osteoclasts, the role of paramyxoviral infection and persistence of the virus in patients with Paget's disease is unclear. Targeting the expression of candidate genes to the cells of osteoclast lineage in transgenic mouse that are permissive to MV infection may allow better understanding of the pathobiology of Paget's disease. It is important to determine a cause and effect relationship for persistence of paramyxoviral infection and genetic predisposition in these patients. Therefore, the etiology of Paget's disease remains uncertain.

REFERENCES

Brandwood CP, Hoyland JA, Hillarby MC, Berry JL, Davies M, Selby PL, Mee AP. 2003. Apoptotic gene expression in Paget's disease: A possible role for Bcl-2. J Pathol 201:504–512.

Cooper C, Schafheutle K, Dennison E, Kellingray S, Guyer P, Barker D. 1999. The epidemiology of Paget's disease in Britain: Is the prevalence decreasing? J Bone Miner Res 14:192–197.

Doyle T, Gunn J, Anderson G, Gill M, Cundy T. 2002. Paget's disease in New Zealand: Evidence for declining prevalence. Bone 31:616–619.

Duran A, Serrano M, Leitges M, Flores JM, Picard S, Brown JP, Moscat J, Diaz-Meco MT. 2004. The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev Cell 6:303–309.

Good DA, Busfield F, Fletcher BH, Duffy DL, Kesting JB, Andersen J, Shaw JT. 2002. Linkage of Paget disease of bone to a novel region on human chromosome 18q23. Am J Hum Genet 70:517–525.

Good DA, Busfield F, Fletcher BH, Lovelock PK, Duffy DL, Kesting JB, Andersen J, Shaw JT. 2004. Identification of SQSTM1 mutations in familial Paget's disease in Australian pedigrees. Bone 35:277–282.

Gordon MT, Mee AP, Anderson DC, Sharpe PT. 1992. Canine distemper virus transcripts sequenced from pagetic bone. Bone Miner 19:159–174.

Gori F, Hofbauer LC, Dunstan CR, Spelsberg TC, Khosla S, Riggs BL. 2000. The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. Endocrinol 141:4768–4776.

Hansen MF, Nellissery MJ, Bhatia P. 1999. Common mechanisms of osteosarcoma and Paget's disease. J Bone Miner Res 14:39–44.

Helfrich MH, Hobson RP, Grabowski PS, Zurbriggen A, Cosby SL, Dickson GR, Fraser WD, Ooi CG, Selby PL, Crisp AJ, Wallace RG, Kahn S, Ralston SH. 2000. A negative search for a paramyxoviral etiology of Paget's disease of bone: Molecular, immunological, and ultrastructural studies in UK patients. J Bone Miner Res 15:2315–2329.

Hocking LJ, Herbert CA, Nicholls RK, Williams F, Bennett ST, Cundy T, Nicholson GC, Wuyts W, Van Hul W, Ralston SH. 2001. Genomewide search in familial Paget's disease of bone shows evidence of genetic heterogeneity with candidate loci on chromosome 2q36, 10q13 and 5q35. Am J Hum Genet 69:1055–1061.

Hocking LJ, Lucas GJ, Daroszewska A, Cundy T, Nicholson GC, Donath J, Walsh JP, Finlayson C, Cavey JR, Ciani B, Sheppard PW, Searle MS, Layfield R, Ralston SH. 2004. Novel UBA domain mutations of SQSTM1 in Paget's disease of bone: Genotype phenotype correlation, functional analysis, and structural consequences. J Bone Miner Res 19:1122–1127.

Hoyland JA, Freemont AJ, Sharpe PT. 1994. Interleukin-6, IL-6 receptor, and IL-6 nuclear factor gene expression in Paget's disease. J Bone Miner Res 9:75–80.

Hughes AE, Ralston SH, Marken J, Bell C, MacPherson H, Wallace RGH, van Hul W, Whyte MP, Nakatsuka K, Hovy L, Anderson DM. 2000. Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis. Nat Genet 24:45–48.

Johnson-Pais TL, Wisdom JH, Weldon KS, Cody JD, Hansen MF, Singer FR, Leach RJ. 2003. Three novel mutations in SQSTM1 identified in familial Paget's disease of bone. J Bone Miner Res 18:1748–1753.

Kurihara N, Reddy SV, Menaa C, Anderson D, Roodman GD. 2000. Osteoclasts expressing the measles virus nucleocapsid gene display a pagetic phenotype. J Clin Invest 105:607–614.

Kurihara N, Reddy SV, Araki N, Ishizuka S, Ozono K, Cornish J, Cundy T, Singer FR, Roodman GD. 2004. Role of TAFII-17, a VDR binding protein, in the increased osteoclast formation in Paget's disease. J Bone Miner Res 19:1154–1164.

Laurin N, Brown JP, Morissette J, Raymond V. 2002. Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget's disease of bone. Am J Hum Genet 70:1582–1588.

Leach RJ, Singer FR, Roodman GD. 2001. The genetics of Paget's disease of the bone. J Clin Endocrinol Metab 86:24–28.

Menaa C, Reddy SV, Barsony J, Cornish J, Cundy T, Roodman GD. 2000a. 1,25 dihydroxy-vitamin D3 hypersensitivity of osteoclast precursors from patients with Paget's disease. J Bone Miner Res 15:1–9.

Menaa C, Reddy SV, Kurihara N, Maeda H, Anderson D, Cundy T, Cornish J, Singer FR, Bruder JM, Roodman GD. 2000b. Enhanced RANK ligand expression and responsivity of bone marrow cells in Paget's disease of bone. J Clin Invest 105:1833–1838.

Mills BG, Singer FR. 1976. Nuclear inclusions in Paget's disease of bone. Science 194:201–202.

Moscat J, Diaz-Meco MT. 2000. The atypical protein kinase Cs. EMBO Rep 1:399–403.

Neale SD, Smith R, Wass JAH, Athanasou NA. 2000. Osteoclast differentiation from circulating mononuclear precursors in Paget's disease is hypersensitive to 1,25-dihydroxyvitamin D3 and RANKL. Bone 27:409–416.

Neale SD, Schulze E, Smith R, Athanasou NA. 2002. The influence of serum cytokines and growth factors on osteoclast formation in Paget's disease. QJM 95:233–240.

Ooi CG, Walsh CA, Gallagher JA, Fraser WD. 2000. Absence of measles virus and canine distemper virus transcripts in long-term bone marrow cultures from patients with Paget's disease of bone. Bone 27:417–421.

Paget J. 1877. On a form of chronic inflammation of bones (osteitis deformans). Med Chir Tr 60:37–63.

Peng KW, Frenzke M, Myers R, Soeffker D, Harvey M, Greiner S, Galanis E, Cattaneo R, Federspiel MJ, Russell SJ. 2003. Biodistribution of oncolytic measles virus after intraperitoneal administration into Ifnar-CD46Ge transgenic mice. Hum Gene Ther 14:1565–1577.

Reddy SV, Singer FR, Roodman GD. 1995. Bone marrow mononuclear cells from patients with Paget's disease contain measles virus nucleocapsid messenger ribonucleic acid that has mutations in a specific region of the sequence. J Clin Endocrinol Metab 80:2108–2111.

Reddy SV, Kurihara N, Menaa C, Roodman GD. 2001a. Paget's disease of bone: A disease of the osteoclast. Rev Endocr Metab Disord 2:195–201.

Reddy SV, Kurihara N, Menaa C, Landucci G, Forthal DF, Koop BA, Windle JJ, Roodman GD. 2001b. Osteoclasts formed by measles virus infected osteoclast precursors from hCD46 transgenic mice express characteristics of pagetic osteoclasts. Endocrinology 142:2898–2905.

Roodman GD, Kurihara N, Ohsaki Y, Kukita A, Hosking D, Demulder A, Smith JF, Singer FR. 1992. Interleukin-6: A potential autocrine/paracrine factor in Paget's disease of bone. J Clin Invest 89:46–52.

Singer FR. 1999. Update on viral etiology of Paget's disease. J Bone Miner Res 14:29–33.

Siris ES. 1999. Paget's disease of bone. In: Favus MJ, editor. Primer on the metabolic bone diseases and disorders of mineral metabolism. Fourth edition. Philadelphia, PA: Lippincott Williams & Wilkins Press. pp 415–425.

Sparks AB, Peterson SN, Bell C, Loftus BJ, Hocking L, Cahill DP, Frassica FJ, Streeten EA, Levine MA, Fraser CM, Adams MD, Broder S, Venter JC, Kinzler KW, Vogelstein B, Ralston SH. 2001. Mutation screening of the TNFRSF11A gene encoding receptor activator of NF kappa B (RANK) in familial and sporadic Paget's disease of bone and osteosarcoma. Calcif Tissue Int 68: 151–155.

Takeshita S, Namba N, Zhao JJ, Jiang Y, Genant HK, Silva MJ, Brodt MD, Helgason CD, Kalesnikoff J, Rauh MJ, Humphries RK, Krystal G, Teitelbaum SL, Ross FP. 2002. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper resorptive osteoclasts. Nat Med 8:943–949.

Tarquini R, Perfetto F, Tarquini B. 1998. Endothelin-1 and Paget's bone disease: Is there a link? Calcif Tissue Int 63:118–120.

Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH, Mumm S. 2002. Osteoprotegerin deficiency and juvenile Paget's disease. New Eng J Med 347:175–184.

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Regulatory Mechanisms Operative in Osteoclasts

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ABSTRACT: The osteoclast is hematopoietic in origin and is the primary bone-resorbing cell derived from monocyte/macrophage lineage. Tumor necrosis factor (TNF) family member, RANK ligand (RANKL) expressed on marrow stromal/osteoblast cells in response to several osteotropic factors, is critical for osteoclast precursor differentiation to form multinucleated osteoclasts, which resorb bone. M-CSF is required for proliferation, survival, and expression of receptor activator of nuclear factor kappa B (RANK) in osteoclast precursors. The interaction of RANKL-RANK results in activation of various signaling cascades during osteoclast development and activation. The osteoclast is an autocrine/paracrine, intracrine regulatory cell that produces factors such as IL-6, Annexin II, TGF-β, OIP-1/hSca, which influence its own formation and activity. In addition, integrin expression in osteoclasts mediate cell-matrix and cell-cell interactions in the bone microenvironment through specific signaling pathways resulting in cytoskeletal organization, polarization, and activation of osteoclasts to resorb bone. Recent molecular genetic studies have identified several transcription factors, such as NF-κB, c-Fos, MITF, and NFATc1, which are essential for osteoclast differentiation. Although a wide variety of molecules, including the reactive oxygen species (ROS) that are differentially regulated during osteoclastogenesis, the precise signal transduction pathways, and molecular mechanisms underlying the gene expression in osteoclasts, are just beginning to be defined. In this review, we discuss the molecular regulatory mechanisms operative during osteoclast differentiation, bone resorption, and survival.

KEY WORDS: osteoclast, differentiation, signaling, transcription factors, bone resorption, tartrate-resistant acid phosphatase, RANKL

I. INTRODUCTION

The osteoclast is the primary bone-resorbing cell, and the majority of evidence favors that it is derived from the monocyte-macrophage lineage. The earliest identifiable osteoclast precursor is the granulocyte-macrophage colony-forming unit (CFU-GM), the granulocyte-macrophage progenitor cells that proliferate and differentiate into committed precursors for the osteoclast. The early precursor differentiates and begins to express CD11b, CD45, and the Kn22 antigen. As these cells differentiate further, they begin to express vitronectin receptors and calcitonin receptors (CTR) (Roodman, 1999). These committed precursors are postmitotic and fuse to form multinucleated osteoclasts. These multinucleated cells (MNC) are then activated to form bone-resorbing osteoclasts and undergo apoptosis. The mature human osteoclast expresses the interleukin-6 receptor, c-fms, the receptor for macrophage colony stimulating factor (M-CSF), vitronectin receptor and calcitonin receptor (CTR). In addition to changes in the surface phenotype that occur during osteoclast differentiation, expression of tartrate-resistant acid phosphatase (TRAP) has been detected in early proliferating osteoclast precursors and is elevated at high levels in committed osteoclast precursors that fuse to form mature osteoclasts. However, the functional role of TRAP in the osteoclast is unclear. Studies using homologous recombination to inactivate

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the TRAP gene have demonstrated that these animals appear to have relatively normal bone resorption and, in fact, have problems with endochondral bone formation and mild osteopetrosis (Hayman et al., 1996). Recently there have been reports that early B-lymphocytes, B220+ cells, can also form osteoclasts (Sato et al., 2001). The molecular and cellular events involved in osteoclast differentiation and the large array of factors regulating osteoclast formation and activity are just beginning to be defined. In this review, the regulatory mechanisms operative during osteoclast development and bone-resorption activity are discussed.

II. MECHANISMS OF OSTEOCLAST DIFFERENTIATION/BONE RESORPTION

RANK ligand (RANKL) is a member of the tumor necrosis factor (TNF) family that is produced by osteoblasts and stromal cells in the bone microenvironment. Receptor activator of NF-κB (RANK) is expressed on committed osteoclast precursors. RANKL in combination with M-CSF induces differentiation of osteoclast precursors/spleen cells to form multinucleated osteoclasts in the absence of stromal/osteoblast cells. RANKL binding to RANK receptor signals through the TRAF6 adapter protein, resulting in activation of NF-κB and c-Jun N-terminal kinase (JNK) in osteoclast precursor cells, which then fuse to form multinucleated osteoclasts (Boyle et al., 2003). It has been suggested that macrophage fusion receptor (MFR) interaction with CD47 and CD44 mediate the adhesion/fusion to form multinucleated osteoclasts (Vignery, 2000). Osteoprotegerin, a decoy receptor of RANKL also called the osteoclastogenesis inhibitory factor, is a member of the TNF receptor superfamily (Simonet et al., 1997). However, recent evidence suggests that osteoprotegerin (OPG) directly induces promatrix metalloproteinase-9 activity and phosphorylation of p38 and ERK1/2 in osteoclast precursor cells. Therefore, it has been suggested that RANKL-RANK-OPG may form a ternary complex on osteoclasts (Theoleyre et al., 2004). Although TRAF6 is essential for both RANKL- and TNF-α–induced osteoclast differentiation, it has been reported that these cytokines did not effectively induce osteoclast precursor cells derived from TRAF5-deficient mice, however, JNK and NF-κB activation occurred in these cells (Kanazawa et al., 2003). These data suggest that other members of TRAF family may also be associated with RANK signaling in osteoclastogenesis. TNF appears to induce both the proliferation and differentiation of osteoclast precursors and activate preformed osteoclasts to resorb bone. TNF-α has been reported to stimulate osteoclast differentiation by a mechanism independent of RANK-RANKL interaction (Kobayashi et al., 2000), but Lam et al. (2000) showed that trace amounts of RANKL are required with TNF-α to promote osteoclastogenesis. Further studies revealed that TNF signals through a TNF type 1 receptor (TNFr1), enhancing the expression levels of c-Src, TRAF2, TRAF6, and MEKK-1 to stimulate osteoclast differentiation. Also, TNF and RANKL synergistically upregulates RANK expression in osteoclast precursors (Zhang et al., 2001).

TRAF6 demonstrates different functional domains that are responsible for osteoclast differentiation and maturation (Kobayashi et al., 2001). Shin et al. (2002) have further identified a novel zinc finger protein, TIZ (TRAF6 inhibitory zinc finger protein) that interacts with N-terminal region of TRAF-6 and thereby inhibits RANKL stimulation of osteoclast differentiation. It has also been shown that RANKL, signaling induce recruitment of SHP-1 (Src homology 2 domain containing phosphatase-1) to TRAF-6 complex, resulting in enhanced IκB-α phosphorylation, degradation, and DNA-binding activity of NF-κB. SHP-1 also regulates RANKL-induced phosphorylation of phosphotidylinositol 3 kinase (PI3K) and Akt (Zhang et al., 2003).

RANKL stimulates p38 activity and phosphorylation of Akt, a downstream target of PI3K and that of extracellular signal-regulated kinase (ERK) suggesting that these molecules may play important roles in osteoclast differentiation (Lee et al., 2002a). Furthermore, expression of the negative form of p38 alpha MAP kinase or MAP kinase kinase (MKK) 6 inhibits RANKL stimulated osteoclast differentiation. It has also been shown that TNF induces osteoclast differentiation and that p38 MAP kinase–deficient mice bone marrow cultures form reduced numbers of osteoclasts compared to control mice (Matsumoto

et al., 2000). RANKL and TNF-α induce phosphorylation of p38 MAPK in osteoclast precursors but not in mature osteoclasts (Li et al., 2002). It has also been demonstrated that differentiation of mouse bone marrow macrophages into osteoclasts in response to RANKL or TNF-α was strongly inhibited by a p38 MAPK inhibitor (Li et al., 2003). These data suggest that a p38 MAP kinase pathway plays an important role in RANKL signaling of osteoclast differentiation, but not for osteoclast function (Lee and Kim, 2003). A schematic illustration of RANKL-RANK interaction and associated signal transduction cascades, which were operative during osteoclast differentiation, bone resorption, and survival, are shown in Figure 1. Recent evidence also indicates that decoy receptor 3 (DcR3) of the TNF receptor superfamily via coupling reverse signaling of ERK and p38 MAPK and stimulating TNF-α synthesis induces osteoclast formation from monocyte/macrophage lineage precursor cells (Yang et al., 2004). Previously it has been demonstrated that Gas6/Tyro 3 (a receptor tyrosine kinase) signaling stimulates mature osteoclasts to resorb bone through p42/p44 MAPK activation (Katagiri et al., 2001).

RANKL has been shown to activate anti-apoptotic serine/threonine kinase Akt/PKB through a signaling complex involving c-Src and TRAF6 leading to phosphorylation of downstream molecules, such as c-Cbl, suggesting a cross talk between TRAF proteins and Src family kinases (Wong et al., 1999). Molecular indexing methods further identified the Jun dimerization protein 2 (JDP2, a member of the AP-1 family), which mediates RANKL-induced osteoclast differentiation (Kawaida et al., 2003). Similarly, a dominant negative form of TAK1 (transforming growth factor-beta-activated kinase 1) has been shown to suppress RANKL-induced AP-1 activation by TRAF2, TRAF5, and TRAF6, and JNK activity, indicating that TAK1 is involved in the RANK signaling through MAPK cascade and NF-κB pathway (Lee et al., 2002b). More recently, it has also been shown that JunB inactivation, specifically in the macrophage-osteoclast lineage, develops osteopetrosis-like phenotype in mice (Kenner et al., 2004).

Recurrent mutation of the gene-encoding ubiquitin-binding protein, sequestosome 1 (SQSTM1/p62), has been reported in patients with Paget's bone disease (Laurin et al., 2002). p62 has been shown to interact with the atypical PKC (aPKC) and TRAF6. More recently, Duran et al. (2004) have shown that RANKL stimulation resulted in upregulation of p62 and formation of a ternary complex of TRAF6, p62, and aPKC during osteoclastogenesis. Furthermore, they have also shown that the genetic inactivation of p62 in mice impairs osteoclastogenesis *in vitro* and *in vivo* through inhibition of IkappaB kinase (IKK) activation and NF-κB nuclear translocation. These data suggest that signaling mechanisms associated with p62 may also play an important role in osteoclast development and function. In addition, protein kinase C-beta has been shown to regulate osteoclast formation and function through participation in the ERK pathway of M-CSF and RANKL signaling (Lee et al., 2003b). Therefore, osteoclast differentiation is a complex process regulated by several signal-transduction cascades and yet to be further delineated to our understanding of downstream effectors and cooperative mechanisms during normal and pathological bone remodeling.

III. TRANSCRIPTION FACTOR/ PROTO-ONCOGENE CONTROL OF OSTEOCLAST DEVELOPMENT/ FUNCTION

In vitro and *in vivo* molecular genetic approaches have provided evidence indicating several transcription factors/proto-oncogenes appear to be the key regulators of osteoclast differentiation and bone-resorption activity (Teitelbaum and Ross, 2003). However, the molecular mechanisms by which transcription factors regulate osteoclast-specific gene expression are just beginning to be defined.

A. NF-κB

NF-κB is an important transcription factor involved in osteoclast differentiation. Mice deficient in both the p50 and p52 subunits of NF-κB develop severe osteopetrosis (Franzoso et al., 1997). Deletion of only the p50 or p52 subunit did not

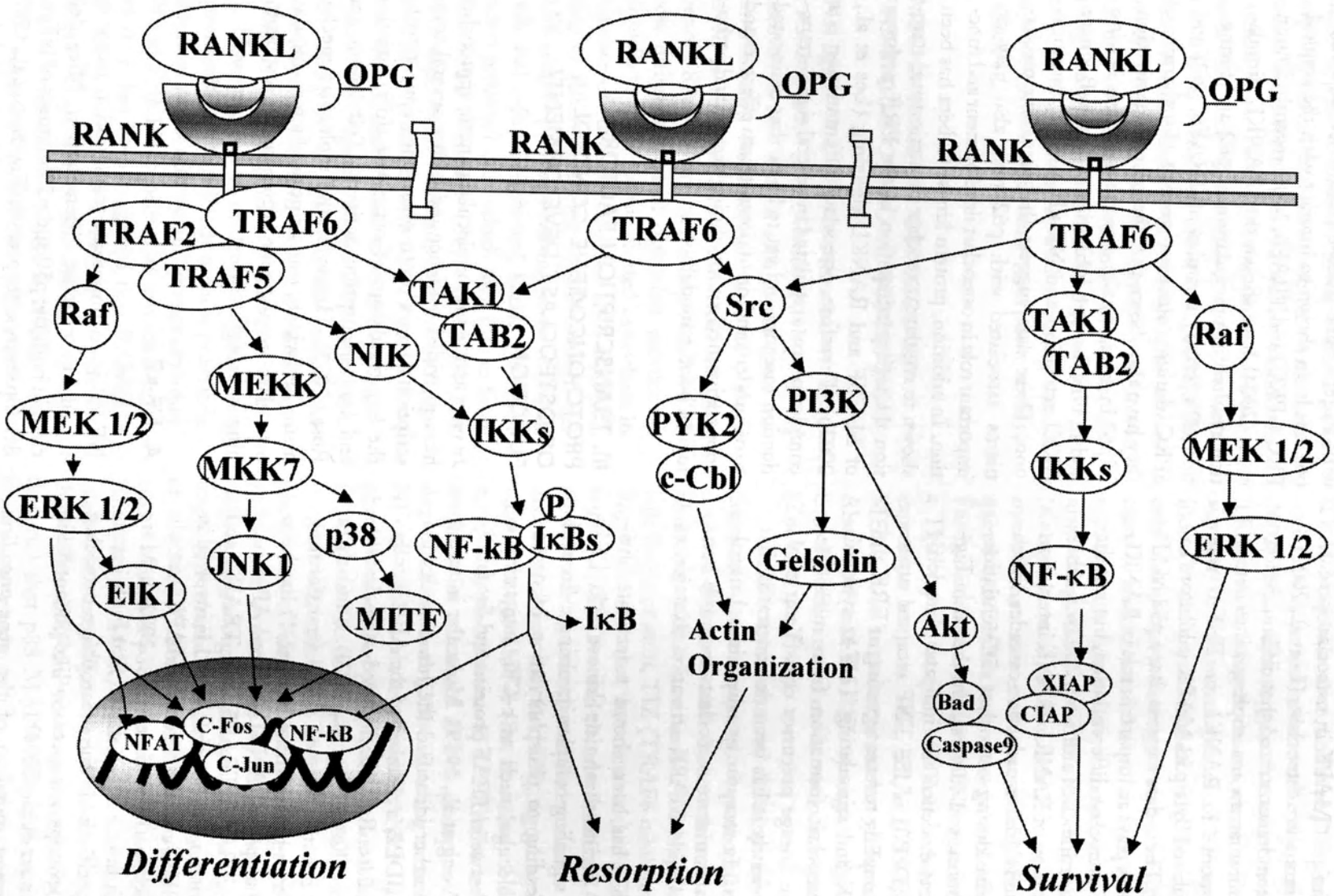

FIGURE 1. RANKL–RANK interaction and associated signaling pathways functional in osteoclasts.

affect bone phenotype. NF-κB plays a critical role in expression of a variety of cytokines involved in osteoclast differentiation, including IL-1, TNF-α, IL-6, GM-CSF, RANKL, and other growth factors. Deletion of both p50 and p52 may affect the production of growth factors critical for osteoclast differentiation as well as RANKL signaling. However, it has been reported that NF-κB p50 and p52 expression is not required for formation of RANK expressing osteoclast progenitors, but is essential for RANK expressing osteoclast precursors to differentiate into osteoclasts in response to RANKL and other osteoclastogenic cytokines (Xing et al., 2002). Recently, it has been demonstrated that RANKL increases expression of the proto-transcription factor p100 and its conversion to p52. NF-κB–inducing kinase (NIK) controls processing of p100 to generate p52, and the deficiency of NIK results in cytosolic accumulation of p100 in osteoclast precursors. In addition, p100-deficient osteoclast precursors showed enhanced sensitivity to RANKL, which further suggests that regulatory mechanisms distinct from the classical IκB-α pathway may be operative in osteoclasts (Novack et al., 2003).

B. PU.1

The myeloid and B cell-specific transcription factor PU.1 is critical for osteoclast differentiation. PU.1 expression is progressively increased as marrow macrophages expressed the osteoclast phenotype *in vitro*. Furthermore, PU.1 expression increased with the induction of osteoclastogenesis by $1,25\text{-}(OH)_2D_3$ and dexamethasone. PU.1 deficient mice are osteopetrotic. The absence of both osteoclasts and macrophages in PU.1 deficient mice suggests that this transcription factor regulates the initial stages of macrophage differentiation (Tondravi et al., 1997). PU.1 has been shown to interact with the microphthalmia transcription factor (MITF) to regulate TRAP gene expression and osteoclast differentiation (Luchin et al., 2001).

C. c-Fos

The AP-1 (Fos/Jun) transcription-factor complex plays important role in skeletal development. c-Fos, a proto-oncogene normally associated with osteosarcomas, also appears to be a key regulator of osteoclast differentiation. Mice lacking the c-Fos proto-oncogene develop osteopetrosis and have normal macrophage differentiation (Wagner and Matsuo, 2003). The Fos-deficient mice have a block in differentiation at the branch point between monocyte-macrophages and osteoclasts, and only form macrophages. Furthermore, transfection of c-Fos cDNA into avian osteoclast precursors induced a two-fold increase in TRAP and osteoclastic bone-resorption activity as compared with controls. These data suggest that prolonged expression of c-Fos can enhance osteoclast differentiation. Overexpression of Fra-1, but not c-Fos, in an immortalized bipotential osteoclast/macrophage precursor cell line caused a significant increase in the proportion of these precursors, which developed CTR and subsequent bone resorption (Owens et al., 1999). These data suggest that Fra-1 may play a role in osteoclast differentiation distinct from that of c-Fos. Fra-1, but none of the Jun proteins (c-Jun, Jun-B, and Jun-D) rescued the block in osteoclast differentiation when the Fra-1 gene is knocked into c-Fos-deficient mice (Fleischmann et al., 2000). The N-terminal portion and the core region of Fos proteins were sufficient for osteoclast differentiation (Wagner and Matsuo, 2003). Recently, these investigators have also shown that RANKL induces transcription of Fosl1 in a c-Fos-dependent manner, thereby establishing a link between RANK signaling and the expression of AP-1 proteins in osteoclast differentiation (Matsuo et al., 2000). c-Fos plays an important role in the proliferative phase of osteoclast progenitors, but not in the terminal differentiation phase or in the bone-resorbing activity of mature osteoclasts (Udagawa et al., 1996).

D. c-Src

c-Src, a proto-oncogene, plays a critical role in the activation of quiescent osteoclasts to become bone-resorbing osteoclasts. Osteoclast formation is normal in animals lacking the c-Src gene. However, they develop osteopetrosis because the osteoclasts are unable to resorb bone (Soriano et al., 1991). These osteoclasts cannot form ruffled borders (Boyce et al., 1992). The substrate for Src

appears to be cortactin, which plays a critical role in the attachment of osteoclasts to bone surfaces. Transgenic mice, which had the wild-type or mutated versions of c-Src proto-oncogene targeted to the osteoclast, demonstrated that expression of the wild-type transgene in only a limited number of tissues can fully rescue the c-Src-deficient phenotype. Interestingly, expression of kinase defective mutants of c-Src in c-Src-deficient mice also reduces osteopetrosis (Schwartzberg et al., 1997). These data suggest that there are essential kinase-independent functions for c-Src *in vivo*. It has been shown that c-Src associated with tubulin only when avian osteoclasts were adherent to bone (Abu-Amer et al., 1997). This would suggest that matrix recognition by osteoclasts induce c-Src to associate with microtubules that mobilize proteins to the cell surface. Tanaka et al. (1996) suggested that the lack of phosphorylation of c-Cbl is the important step in osteoclast activation in c-Src–deficient animals. Also, c-Cbl forms molecular complexes with Src and Pyk2 to regulate osteoclast adhesion and motility (Sanjay et al., 2001). More recently, Miyazaki et al. (2004) reported that Src kinase activity is essential for osteoclast function. They have concluded that phosphorylation of Cbl and the subsequent recruitment and activation of PI3K may be a critical signaling event downstream of Src in osteoclasts. These workers have previously identified the Tax transcription factor, which has a role in commitment of cells to the osteoclast lineage and/or osteoclast differentiation (Inoue et al., 1997).

E. MITF

The microphthalmia transcription factor (MITF) is required for terminal osteoclast differentiation. Mice lacking the MITF gene developed osteopetrosis. Mansky et al. (2002a) have shown that MITF is involved in the RANKL-signaling pathway and phosphorylation of MITF results in activation of target genes in osteoclasts. M-CSF induces phosphorylation of MITF, and that MITF and the TFE3 transcription factors are required for osteoclast gene activation (Weilbacher et al., 2001). MITF and TFE3 are closely related helix-loop-helix transcription factors, which have been implicated in osteoclast development and function. MITF and the related helix-loop-helix factor TFE-3 and TFE-C have been shown to collaborate to activate the TRAP gene promoter during osteoclast differentiation (Mansky et al., 2002b). Furthermore, the constitutively active Rac1 or MKK6 gene could collaborate with MITF to activate TRAP gene transcription (Mansky et al., 2002a). These results indicate that MITF is a target for the RANKL signaling in osteoclasts and that phosphorylation of MITF leads to an increase in osteoclast-specific gene expression.

F. c-Myc

Recently, it has been reported that RANKL induces c-Myc proto-oncogene expression during osteoclast differentiation of RAW 264.7 cells (Battaglino et al., 2002). Furthermore, expression of a dominant negative Myc in these cells results in a blockade of RANKL-induced osteoclast formation. In contrast, c-Myc has been shown to negatively regulate TRAP gene transcription in osteoclasts (Daumer et al., 2002). These data suggest that c-Myc plays important role in regulation of target gene expression and RANKL-induced differentiation of osteoclasts.

G. NFAT

Recently, it has been reported that the calcineurin/NFAT (nuclear factor of activated T cells) signaling pathway regulates osteoclast differentiation. Immunosuppressant drugs, such as cyclosporin A, act as specific inhibitors of the calcium-/calmodulin-regulated serine/threonine phosphatase, calcineurin. RANKL signaling induced elevation of cytosolic Ca^{2+}, which resulted in activation of calcineurin. Activated calcineurin dephosphorylate cytosolic NFAT is followed by nuclear translocation and interaction with the AP-1 transcription factor complex to modulate target gene expression in osteoclasts. Phosphorylation of NFATc by glycogen synthase kinase-3 inhibits the ability of NFATc to bind DNA (Hogan et al., 2003). NFATc1-deficient embryonic stem cells fail to differentiate into bone-resorbing osteoclasts in response to RANKL stimulation (Takayanagi et al., 2002b). Furthermore, RANKL has been

shown to upregulate NFATc1 expression during osteoclast differentiation. These results also indicated that calcineurin is an essential downstream effector of the RANKL-signaling pathway. In addition, the constitutively active calcineurin-independent NFATc1 mutant expression is sufficient to induce osteoclast differentiation of RAW264.7 cells *in vitro* (Hirotani et al., 2004). Therefore, NFATc1 plays an important role in regulating osteoclast differentiation and function in response to RANKL stimulation (Matsuo et al., 2004; Ikeda et al., 2004).

IV. INTEGRIN SIGNALING AND OSTEOCLAST ADHESION

Kania et al. (1997) determined that CD44, a cell-surface glycoprotein, which is known to function as an adhesion receptor, is also involved in osteoclast differentiation. Monoclonal antibodies against CD44 inhibit osteoclast formation. However, CD44 antibodies did not inhibit the bone-resorptive activity of mature osteoclasts, suggesting that this surface antigen play a role predominantly in osteoclast formation. More recently, Chellaiah et al. (2003) reported that Rho kinase is a potent activator of CD44 surface expression. Furthermore, they have identified cooperativity between $\alpha_v\beta_3$ integrins and CD44 for osteoclast motility and bone resorption. Integrins are heterodimeric adhesion receptors that mediate cell-matrix and cell-cell interactions. Osteoclasts highly express the $a_v\beta_3$ vitronectin receptors that bind to several extracellular matrix proteins, including vitronectin, osteopontin, and bone sialoprotein. Furthermore, evidence is acumulated that several signaling and adaptor molecules are involved in $a_v\beta_3$ integrin-dependent signaling pathways, which include PI3K, c-Src, PYK2, FAK, and $p130^{Cas}$. In addition, cytoskeletal molecules, such as paxillin vinculin, gelsolin, and F-actin, are recruited to adhesion contacts upon integrin activation (Duong et al., 2000). PYK2 and focal adhesion kinase (FAK) are major tyrosine kinases activated by integrin engagement. Studies have shown that ligation of $\alpha_v\beta_3$ integrin in osteoclasts induces c-Src-dependent tyrosine phosphorylation and Proline-rich tyrosine kinase 2 (PYK2) activation, leading to a cytoskeletal rearrangement, migration, and polarization of osteoclasts to resorb bone. However, PYK2 is the main adhesion-induced kinase in bone-resorbing osteoclasts. Further studies demonstrated that PYK2 autophosphorylation, but not kinase activity is essential in the regulation of adhesion-dependent cytoskeletal organization in osteoclasts (Lakkakorpi et al., 2003). Recently, it has been reported that β_3 integrin null osteoclasts are dysfunctional, however, the osteoclast numbers are increased *in vivo*. *In vitro*, addition of M-CSF, but not RANKL, completely rescues $\beta_3^{-/-}$ osteoclastogenesis. These studies have also delineated that c-Fms and $\alpha_v\beta_3$ collaborate through shared activation of the ERK/c-Fos signaling pathway during the osteoclastogenesis (Faccio et al., 2003a). It has also been demonstrated that M-CSF induces c-Cbl phosphorylation independent of cell adhesion, however, phosphorylation of $p130^{Cas}$ depends on $\alpha_v\beta_3$ integrin mediated cell adhesion. Therefore, $p130^{Cas}$ and c-Cbl play distinct roles in the signal transduction pathways that mediate cytoskeletal organization in osteoclasts (Nakamura et al., 2003). Recently, it has also been found that leupaxin, a cytoskeleton adaptor protein that shares homology with the focal adhesion protein paxillin, is associated with PYK2 and pp125FAK in the osteoclast (Gupta et al., 2003). Therefore, leupaxin may be a critical component of the osteoclast podosomal signaling complex.

In addition, Wiskott Aldrich Syndrome protein (WASp) has shown to be involved in the proliferation and differentiation of $CD34^+$ hemopoietic progenitor cells. More recently, WASp has been shown to be a critical component of podosomes in osteoclasts and that WASp deficiency in mice results in failure to form osteoclast-sealing zones and defects in bone resorption (Calle et al., 2004). Therefore, WASp may function through participation in signal transduction and in the regulation of the cytoskeleton during osteoclast bone resorption.

V. AUTOCRINE/PARACRINE REGULATION OF OSTEOCLASTOGENESIS

Osteoclast is an abundant source of cytokines in the bone microenvironment. High levels of inter-

leukin-6 (IL-6) have been associated with several bone diseases, including Paget's disease, multiple myeloma, osteoporosis, and Gorham-Stout disease. Furthermore, IL-6 can act as an autocrine/paracrine factor that stimulates osteoclast formation in human marrow cultures in the absence of added IL-6 receptors (Roodman, 1999). We have also demonstrated that addition of a neutralizing antibody to IL-6 or antisense deoxyoligonucleotides to IL-6 mRNA can block bone resorption by human osteoclasts isolated from giant cell tumors of bone (Reddy et al., 1994). However, osteoblastic cells from transgenic mice constitutively overexpressing human IL-6 receptors demonstrated that the ability of IL-6 to induce osteoclast differentiation depended on signal transduction mediated by IL-6 receptors expressed on osteoblastic cells, but not on osteoclast progenitors (Udagawa et al., 1995).

Oursler (1994) has shown that osteoclasts produce TGF-β, which can inhibit or enhance osteoclast formation and bone resorption, depending on its concentration. Chenu et al. (1988) also have showed that TGF-β inhibits both the proliferation and fusion of human osteoclast precursors. In addition, TGF-β appears to preferentially induce granulocytic rather than monocyte-macrophage differentiation of early monocyte precursors. These data demonstrate that TGF-β, in addition to inhibiting all stages of osteoclast differentiation, also appear to deplete the precursor pool for osteoclasts by shifting the differentiation of immature precursor cells toward the granulocytic lineage. Recent studies have shown that very low concentrations of TGF-β1 can enhance osteoclast formation and bone resorption (Yan et al., 2001). It has also been shown that TGF-β promotes human osteoclastogenesis through stimulation of the p38 MAPK and that continuous exposure to TGF-β abrogates osteoclastogenesis through downregulation of RANK expression in osteoclast precursor cells (Karsdal et al., 2003). Furthermore, TGF-β upregulates suppressors of cytokine signaling, SOCS-3 in osteoclast precursors opposing the inhibitory cytokine signaling for osteoclast commitment (Fox et al., 2003). More recently, TGF-β in the presence of M-CSF has been shown to directly induce osteoclast precursors to differentiate into bone-resorbing osteoclasts (Itonaga et al., 2004). These results suggest that the effects of TGF-β on osteoclast activity differ, depending on the amount of TGF-β present in the bone microenvironment.

We have previously used an expression-cloning approach with a human osteoclast cDNA library and identified Annexin II, which stimulates osteoclast formation. We further demonstrated that Annexin II enhanced GM-CSF production by bone marrow T-lymphocytes and stromal cells, increasing the proliferation of osteoclast precursors and resulting in increased osteoclastogenesis. We also identified osteoclast inhibitory peptide-1 (OIP-1/hSca), which inhibits osteoclast development and bone resorption (Reddy and Roodman, 1998). More recently, we have shown that interferon-γ (IFN-γ) enhances OIP-1/hSca expression and that OIP-1 inhibits osteoclast formation through suppression of TRAF2 expression and JNK activity in RANKL-stimulated osteoclast precursor cells (Koide et al., 2003). In addition, we have identified a novel intracellular signaling molecule termed osteoclast stimulatory factor (OSF) which interacts with c-Src and the spinal muscular atrophy (SMA) disease-determining gene product (SMN), resulting in the release of soluble factors that enhance osteoclast differentiation (Kurihara et al., 2001). Similarly, Roodman and coworkers, using cDNA subtractive hybridization methods, demonstrated that the eosinophil chemotactic factor-L (ECF-L), ADAM8 (a disintegrin and metalloproteinase) is highly expressed in mature osteoclasts. ECF-L acted at the later stages of osteoclast formation and increased migration of osteoclast precursors. ADAM8 affects the later stages of osteoclast differentiation, increasing bone-resorption capacity of these cells. They have also previously identified legumain as an inhibitor of osteoclast formation and bone resorption (Oba et al., 2003; Choi et al., 2001; Roodman, 1999).

Immune cell products (such as IFNs), which are released in response to inflammatory stimuli or viral infections, are negative regulators for bone remodeling. In addition, IFNs inhibit CFU-GM growth and recruitment of osteoclast precursors to fuse and form multinucleated osteoclasts. T-cell production of IFN-γ also strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway (Takayanagi et al., 2000). IFN-γ induces rapid degradation of the RANK

adaptor protein, TRAF6, which results in strong inhibition of the RANKL-induced NF-κB activation and c-Jun kinase activity. These studies suggested that there is cross talk between TNF and IFN families of cytokines, through which IFN provides a negative link between T-cell activation and bone resorption. IFN-γ exerts a direct effect on osteoclast progenitors, and TGF-β antagonizes the effect of IFN-γ (Fox and Chambers, 2000). Although the mechanism of glucocorticoid action on osteoclasts is unclear, it has been reported that dexamethasone enhances osteoclast formation synergistically with TGF-β by priming the osteoclast precursor differentiation. Dexamethasome decreased IFN-β production in osteoclasts, resulting in an increased rate of osteoclastogenesis (Takuma et al., 2003). Recently, it has been shown that RANKL induces the IFN-β gene in osteoclast precursor cells and that IFN-β inhibits osteoclast differentiation by interfering with the RANKL-induced expression of c-Fos to maintain bone homeostasis (Takayanagi et al., 2002a). Similarly, IL-7 has been shown to be a direct inhibitor of osteoclastogenesis *in vitro* and that IL-7-deficient mice-derived bone marrow cultures form increased numbers of osteoclasts in response to several osteotropic factors, such as vitamin D3, PTH, M-CSF, and RANKL. In contrast, bone marrow cultures derived from IL-7 receptor null mice form decreased numbers of osteoclasts when stimulated with these cytokines, further suggesting that IL-7 may have complex regulatory mechanisms signaling through multiple receptors expressed on osteoclast precursor cells (Lee et al., 2003a). Osteoclasts have been shown to secrete the chemotactic cytokine mim-1, however, the signaling mechanisms by which mim-1 affect osteoclastogenesis are unknown (Falany et al., 2001).

Calcitonin is a peptide hormone secreted by the parafollicular cells of the thyroid gland and a potent inhibitor of osteoclastic bone resorption. Calcitonin receptors (CTR) are expressed on committed osteoclast precursors and mature osteoclasts. Calcitonin downregulates expression of CTR in osteoclast precursors and mature osteoclasts (Takahashi et al., 1995). CTR genes demonstrated alternative splicing of transcripts and osteoclast specificity (Anusaksathien et al., 2001). Calcitonin acts on osteoclasts by stimulating adenylcyclase activity and cAMP accumulation, which results in immobilization of the osteoclast and contraction of the osteoclast away from the bone surface (Gorn et al., 1995). Osteoclasts continuously exposed to calcitonin can escape the effects of calcitonin. The mechanism responsible for this escape phenomenon is unclear, but may be due to the effects of calcitonin on transcriptional regulation of CTR gene expression and downregulation of CTR on the surface of the osteoclast.

VI. MECHANISMS OF OSTEOCLAST SURVIVAL/APOPTOSIS

Differentiated osteoclasts undergo rapid apoptosis. Osteoclast cultures stimulated with RANKL and M-CSF indicated that both the MEK/ERK and AKT/NF-κB pathways contribute to osteoclast survival. The inhibition of PI3K, which activates either of these pathways, caused apoptosis through blockade of MEK1/2, ERK1/2, and AKT phosphorylation and NF-κB activation in purified osteoclasts. This suggests that PI3K coordinately activates two distinct survival pathways that are important for osteoclast survival (Gingery et al., 2003). It has been reported that IL-1 alpha strongly induced ERK and NF-κB activation. However, ERK activation is responsible for osteoclast survival (Miyazaki et al., 2000). Similarly, TNF-α has been shown to promote osteoclast survival by suppression of caspase activation through PI3K, AKT, and MEK/ERK signaling pathways (Lee et al., 2001).

M-CSF promotes proliferation and survival of osteoclast precursors. A frame-shift mutation in the coding region of the M-CSF gene in op/op mice results in an osteopetrotic phenotype due to the lack of functionally active M-CSF (Yoshida et al., 1990). M-CSF downregulates c-fms expression and the entry of osteoclast progenitors into the osteoclast lineage in murine bone marrow cultures (Fan et al., 1997). Osteopetrosis in op/op mice improves with age, suggesting that M-CSF is required for osteoclast development in younger animals (Nilsson et al., 1995). Therefore, M-CSF produced by osteoblasts appears to be indispensable for proliferation and differentiation of osteoclast progenitors, but can be substituted by other factors. M-CSF has been shown to induce RANK expression in osteoclast precursor cells (Arai et al.,

1999). PI3K has been shown to be a critical downstream effector from c-fms, $\alpha_v\beta_3$ integrin, and RANK. M-CSF stimulates osteoclast spreading, motility, and cytoskeletal organization. PI3K interacts with c-Src in mediating the effects of activated c-fms (Golden and Insogna, 2004). In addition, GM-CSF in the presence of IL-6 or TNF-α increased osteoclast numbers beyond that seen with either IL-6 or TNF-α alone (Gorny et al., 2004). Recently, RANKL, M-CSF, and TNF-α have also been shown to promote osteoclast survival by signaling through mammalian target of rapamycin, mTOR/S6 kinase activation (Glantschnig et al., 2003). Lacey et al. (2000) have shown that RANKL is essential, but not sufficient for osteoclast survival, and endogenous CSF-1 levels are insufficient to maintain osteoclast viability in the absence of RANKL. Kojima et al. (2001) further demonstrated that RANKL inhibit rDrak1, a kinase that is highly expressed in active osteoclasts and induces apoptosis. It has also been shown that RANKL activate JNK1, but not JNK2, and that JNK1 protects bone-marrow monocytes from RANKL-induced apoptosis during differentiation. JNK1 activation modulates osteoclast differentiation through both c-Jun phosphorylation-dependent and -independent mechanisms (David et al., 2002). RANKL has been shown to act through phospholipase C to release Ca^{2+} from intracellular stores, accelerating nuclear translocation of NF-κB and promotes osteoclast survival (Komarova et al., 2003). Furthermore, overexpression of PTEN, a tumor-suppressor gene, blocked RANKL-activated Akt-stimulated survival (Sugatani et al., 2003). Osteoclast apoptosis is also associated with rapid increase in the proapoptotic Bcl-2 family member Bim. Osteoclasts formed in $Bim^{-/-}$ mice-derived bone marrow cultures showed a marked increase in survival rate in the absence of M-CSF, however, bone-resorbing activity of these osteoclasts appeared to be significantly reduced compared to normal osteoclasts. These studies have shown that ubiquitylation of Bim regulates osteoclast apoptosis and activation (Akiyama et al., 2003). Nitric oxide production in the osteoclast promotes apoptosis. Colony-stimulating factor-1 (CSF-1), IL-1 beta, and RANKL protected against the apoptosis through inhibition of conversion of procaspases-3 and -9 to their mature forms. Furthermore, CSF-1 induces expression of endogenous caspase inhibitor protein, X-linked inhibitor of apoptosis (XIAP) in osteoclasts (Kanaoka et al., 2000). Xing et al. (2001) has also demonstrated that transgenic expression of truncated Src, which lacks the kinase domain, induced osteoclast apoptosis. These data provide evidence that Src family kinases are required *in vivo* for survival of osteoclasts associated with RANKL-RANK receptor signaling. Recently, it has been shown that expression of Fas death receptors is upregulated during osteoclast differentiation. Fas-mediated osteoclast apoptosis involves mitochondrial release of cytochrome *c* and activation of caspases 3 and 9 analogous to other cell systems (Wu et al., 2003). The expression of flotillin, a membrane raft component, has been shown to increase during osteoclastogenesis. Furthermore, disruption of rafts blocked RANK signaling of TRAF6 translocation and Akt activation, reducing the survival of osteoclasts (Ha et al., 2003). It has been reported that lipopolysaccharides acts through Toll-like receptors (TLR-4) in osteoclasts promoting survival (Itoh et al., 2003). Furthermore, lipoproteins have been shown to modulate osteoclast survival (Luegmayr et al., 2004). These data suggest that regulation of osteoclast apoptosis plays an important role in the normal bone-remodeling process to either enhance or inhibit osteoclastic bone resorption.

VII. OTHER SIGNALING MECHANISMS FUNCTIONAL IN OSTEOCLASTS

Reactive oxygen species, such as superoxide production, contribute to osteoclastic bone resorption. Evidence also indicates that the NADPH oxidase system is responsible for superoxide generation in osteoclasts (Steinbeck et al., 1994). Yang and coworkers (2001) have cloned and characterized Nox 4, a novel NAPDH oxidase subunit that is highly expressed and functional in generating superoxide in osteoclasts. It has also been proposed that proteins containing redox-active iron, such as TRAP, highly expressed in resorbing osteoclasts generate reactive oxygen species representing a novel mechanism of intracellular fragmentation of bone-resorption products (Halleen et al., 1999). However, it is unclear the signaling mechanisms by which superoxide affects osteoclast bone-resorption activity in normal and patho-

logical conditions, such as Paget's bone disease and osteopetrosis. Similarly, nitric oxide (NO) generated from L-arginine by the enzyme nitric oxide synthase (NOS) is a unique inter- and intracellular signaling molecule associated with osteoclast function. Targeted disruption of three isoforms of NOS indicated that NOS I mediate osteoclast activity *in vitro* and *in vivo* (Jung et al., 2003). Recent evidence also suggests that osteoclasts express functional P2X4 and P2X7 receptors, which are ATP-gated cation channels, and that extracellular nucleotides act through P2X7 receptors to active NF-κB to regulate osteoclast formation and activity (Korcok et al., 2004). In addition, it has been shown that ryanodine-receptor (RyR) type II isoform expressed on a plasma membrane functions as an extracellular Ca^{2+} sensor. However, the molecular-signaling mechanisms coordinating RyR expression in osteoclast nuclear and plasma membranes and Ca^{2+} influx are yet to be delineated (Zaidi et al., 2004).

Evidence also suggests that several other signaling molecules also play an important role in osteoclast development and function. SHIP (Src homology 2-containing inositol-5-phosphatase) has been shown to be a negative regulator of osteoclast formation and activity. SHIP-deficient mice are severely osteoporotic due to increased number of hyperresorptive osteoclasts (Takeshita et al., 2002). More recently, it has also been reported that osteoclats express a recently described signaling adapter protein, DAP12, and associated receptor (DAR). Deficiency of DAP12 is associated with bone abnormalities in both mice and humans, and it further indicated that DAP12 signaling regulates formation of multinucleated osteoclasts (Humphrey et al., 2004). However, a high dose of M-CSF has been shown to partially rescue the $DAP12^{-/-}$ osteoclast phenotype (Faccio et al., 2003b). Also, the deficiency of TREM-2, an immunoglobulin-like cell surface receptor associated with DAP12, results in formation of immature osteoclasts with an impaired bone resorptivity (Cella et al., 2003).

In addition, gene array analysis further identified several genes, regulated by RANKL, which include cytokines and cytokine receptors (RANTES and CSF2R-α) and transcription factors (NFATc1, GABP-α, c-Jun, and FUSE-binding protein 1) during osteoclast differentiation of human peripheral blood mononuclear cells (Day et al., 2004). Similarly, oligonucleotide microarray analysis further identified M-CSF–induced expression of several signaling molecules (TRAF2A, PI3K, MEKK3, RIPK1), interleukins, interferons, and their receptors (IL-1α, IL-18, IFN-β, IFN-γR) during osteoclast differentiation (Cappellen et al., 2002). Although a wide variety of molecules that are differentially regulated during osteoclastogenesis have been identified, the associated signal transduction cascades, regulating osteoclast formation and bone resorption activity, are just beginning to be defined.

VIII. SUMMARY

Osteoclast differentiation is a complex process that is regulated by autocrine/paracrine and intracrine signaling mechanisms. RANKL and M-CSF, produced by stromal/osteoblast cells in the marrow microenvironment, play critical roles in osteoclast precursor cell differentiation to form multinucleated osteoclasts. Transcription factors (such as PU.1, c-Fos, NF-κB, and NFAT), which modulate target gene expression, are critical for osteoclast development and bone-resorption activity. Identification of signaling molecules and differential gene expression associated with RANKL-induced osteoclast differentiation should provide further insight into the complex regulatory mechanisms of osteoclast survival, differentiation, and bone resorption. These studies may identify potential therapeutic molecular targets to modulate osteoclast formation and activity in pathologic conditions with abnormal bone remodeling.

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REFERENCES

Abu-Amer Y, Ross FP, Schlesinger P, Tondravi MM, Teitelbaum SL (1997): Substrate recognition by osteo-

clast precursors induces C-src/microtubule association. J Cell Biol 137:247–258.

Akiyama T, Bouillet P, Miyazaki T, Kadono Y, Chikuda H, Chung UI, Fukuda A, Hikita A, Seto H, Okada T, Inaba T, Sanjay A, Baron R, Kawaguchi H, Oda H, Nakamura K, Strasser A, Tanaka S (2003): Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. EMBO J 22:6653–6664.

Anusaksathien O, Laplace C, Li X, Ren Y, Peng L, Goldring SR, Galson DL (2001):Tissue specific and ubiquitous promoters direct the expression of alternatively spliced transcripts from the calcitonin receptor gene. J Biol Chem 276:22663–22674.

Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T (1999): Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 190:1741–1754.

Battaglino R, Kim D, Fu J, Vaage B, Fu XY, Stashenko P (2002): c-myc is required for osteoclast differentiation. J Bone Miner Res 17:763–773.

Boyce BF, Yoneda T, Lowe C, Soriano P, Mundy GR (1992): Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. J Clin Invest 90:1622–1627.

Boyle WJ, Simonet WS, Lacey DL (2003): Osteoclast differentiation and activation. Nature 423:337–342.

Calle Y, Jones GE, Jagger C, Fuller K, Blundell MP, Chow J, Chambers T, Thrasher AJ (2004): WASp deficiency in mice results in failure to form osteoclast sealing zones and defects in bone resorption. Blood 103:3552–3561.

Cappellen D, Luong-Nguyen NH, Bongiovanni S, Grenet O, Wanke C, Susa M (2002): Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFkappa B. J Biol Chem 277:21971–21982.

Cella M, Buonsanti C, Strader C, Kondo T, Salmaggi A, Colonna M (2003): Impaired differentiation of osteoclasts in TREM-2-deficient individuals. J Exp Med 198:645–651.

Chellaiah MA, Biswas RS, Rittling SR, Denhardt DT, Hruska KA (2003): Rho-dependent Rho kinase activation increases CD44 surface expression and bone resorption in osteoclasts. J Biol Chem 278:29086–29097.

Chenu C, Pfeilschifter J, Mundy GR, Roodman GD (1988): Transforming growth factor beta inhibits formation of osteoclast-like cells in long-term human marrow cultures. Proc Natl Acad Sci U S A 85:5683–5687.

Choi SJ, Han JH, Roodman GD (2001): ADAM8: a novel osteoclast stimulating factor. J Bone Miner Res 16:814–822.

Daumer KM, Taparowsky EJ, Hall DJ, Steinbeck MJ (2002): Transcription from the tartrate-resistant acid phosphatase promoter is negatively regulated by the Myc oncoprotein. J Bone Miner Res 17:1701–1709.

David JP, Sabapathy K, Hoffmann O, Idarraga MH, Wagner EF (2002): JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and independent mechanisms. J Cell Sci 115:4317–4325.

Day CJ, Kim MS, Stephens SR, Simcock WE, Aitken CJ, Nicholson GC, Morrison NA (2004): Gene array identification of osteoclast genes: Differential inhibition of osteoclastogenesis by cyclosporin A and granulocyte macrophage colony stimulating factor. J Cell Biochem 91:303–315.

Duong LT, Lakkakorpi P, Nakamura I, Rodan GA (2000): Integrins and signaling in osteoclast function. Matrix Biol 19:97–105.

Duran A, Serrano M, Leitges M, Flores JM, Picard S, Brown JP, Moscat J, Diaz-Meco MT (2004): The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev Cell 6:303–309.

Faccio R, Takeshita S, Zallone A, Ross FP, Teitelbaum SL (2003a): c-Fms and the alphavbeta3 integrin collaborate during osteoclast differentiation. J Clin Invest 111: 749–758.

Faccio R, Zou W, Colaianni G, Teitelbaum SL, Ross FP (2003b): High dose M-CSF partially rescues the Dap12–/– osteoclast phenotype. J Cell Biochem 90: 871–883.

Falany ML, Thames AM, McDonald JM, Blair HC, McKenna MA, Moore RE, Young MK, Williams JP (2001): Osteoclasts secrete the chemotactic cytokine mim-1. Biochem Biophys Res Commun 281:180–185.

Fan X, Biskobing DM, Fan D, Hofstetter W, Rubin J (1997): Macrophage colony stimulating factor downregulates MCSF-receptor expression and entry of progenitors into the osteoclast lineage. J Bone Miner Res 12:1387–1395.

Fleischmann A, Hafezi F, Elliott C, Reme CE, Ruther U, Wagner EF (2000): Fra-1 replaces c-Fos-dependent functions in mice. Genes Dev 14:2695–2700.

Fox SW, Chambers TJ (2000): Interferon-gamma directly inhibits TRANCE-induced osteoclastogenesis. Biochem Biophys Res Commun 276:868–872.

Fox SW, Haque SJ, Lovibond AC, Chambers TJ (2003): The possible role of TGF-beta-induced suppressors of cytokine signaling expression in osteoclast/macrophage lineage commitment *in vitro*. J Immunol 170:3679–3687.

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonardi A, Tran T, Boyce BF, Siebenlist U (1997): Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev 11:3482–3496.

Gingery A, Bradley E, Shaw A, Oursler MJ (2003): Phosphatidylinositol 3-kinase coordinately activates the MEK/ERK and AKT/NFkappaB pathways to maintain osteoclast survival. J Cell Biochem 89:165–179.

Glantschnig H, Fisher JE, Wesolowski G, Rodan GA, Reszka AA (2003): M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Cell Death Differ 10:1165–1177.

Golden LH, Insogna KL (2004): The expanding role of PI3-kinase in bone. Bone 34:3–12.

Gorn AH, Rudolph SM, Flannery MR, Morton CC, Weremowicz S, Wang TZ, Krane SM, Goldring SR (1995): Expression of two human skeletal calcitonin receptor isoforms cloned from a giant cell tumor of bone: The first intracellular domain modulates ligand binding and signal transduction. J Clin Invest 95:2680–2691.

Gorny G, Shaw A, Oursler MJ (2004): IL-6, LIF and TNF-alpha regulation of GM-CSF inhibition of osteoclastogenesis *in vitro*. Exp Cell Res 294:149–158.

Gupta A, Lee BS, Khadeer MA, Tang Z, Chellaiah M, Abu-Amer Y, Goldknopf J, Hruska KA (2003) Leupaxin is a critical adaptor protein in the adhesion zone of the osteoclast. J Bone Miner Res 18:669–685.

Ha H, Kwak HB, Lee SK, Na DS, Rudd CE, Lee ZH, Kim HH (2003): Membrane rafts play a crucial role in receptor activator of nuclear factor kappaB signaling and osteoclast function. J Biol Chem 278:18573–18580.

Halleen JM, Raisanen S, Salo JJ, Reddy SV, Roodman GD, Hentunen TA, Lehenkari PP, Kaija H, Vihko P, Vaananen HK (1999): Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. J Biol Chem 274:22907–22910.

Hayman AR, Jones SJ, Boyde A, Foster D, Colledge WH, Carlton MB, Evans MJ, Cox TM (1996): Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. Development 122:3151–3162.

Hirotani H, Tuohy NA, Woo JT, Stern PH, Clipstone NA (2004): The calcineurin/NFAT signaling pathway regulates osteoclastogenesis in RAW264.7 cells. J Biol Chem 279:13984–13992.

Hogan PG, Chen L, Nardone J, Rao A (2003): Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17:2205–2232.

Humphrey MB, Ogasawara K, Yao W, Spusta SC, Daws MR, Lane NE, Lanier LL, Nakamura MC (2004): The signaling adapter protein DAP12 regulates multinucleation during osteoclast development. J Bone Miner Res 19:224–234.

Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, Hata K, Yamashita K, Hiraga T, Watanabe T, Kukita T, Yoshioka K, Rao A, Yoneda T (2004): Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J Clin Invest 114:463–465, 2004.

Inoue D, Santiago P, Horne WC, Baron R (1997): Identification of an osteoclast transcription factor that binds to the human T cell leukemia virus type I-long terminal repeat enhancer element. J Biol Chem 272: 25386–25393.

Itoh K, Udagawa N, Kobayashi K, Suda K, Li X, Takami M, Okahashi N, Nishihara T, Takahashi N (2003): Lipopolysaccharide promotes the survival of osteoclasts via Toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. J Immunol 170:3688–3695.

Itonaga I, Sabokbar A, Sun SG, Kudo O, Danks L, Ferguson D, Fujikawa Y, Athanasou NA (2004): Transforming growth factor-beta induces osteoclast formation in the absence of RANKL. Bone 34:57–64.

Jung JY, Lin AC, Ramos LM, Faddis BT, Chole RA (2003): Nitric oxide synthase I mediates osteoclast activity *in vitro* and *in vivo*. J Cell Biochem 89:613–621.

Kanaoka K, Kobayashi Y, Hashimoto F, Nakashima T, Shibata M, Kobayashi K, Kato Y, Sakai H (2000): A common downstream signaling activity of osteoclast survival factors that prevent nitric oxide-promoted osteoclast apoptosis. Endocrinology 141:2995–3005.

Kanazawa K, Azuma Y, Nakano H, Kudo A (2003): TRAF5 functions in both RANKL and TNFalpha-induced osteoclastogenesis. J Bone Miner Res 18:443–450.

Kania JR, Kehat-Stadler T, Kupfer SR (1997): CD44 antibodies inhibit osteoclast formation. J Bone Miner Res 12:1155–1164.

Karsdal MA, Hjorth P, Henriksen K, Kirkegaard T, Nielsen KL, Lou H, Delaisse JM, Foged NT (2003): Transforming growth factor-beta controls human osteoclastogenesis through the p38 MAPK and regulation of RANK expression. J Biol Chem 278:44975–44987.

Katagiri M, Hakeda Y, Chikazu D, Ogasawara T, Takato T, Kumegawa M, Nakamura K, Kawaguchi H (2001): Mechanism of stimulation of osteoclastic bone resorption through Gas6/Tyro 3, a receptor tyrosine kinase signaling, in mouse osteoclasts. J Biol Chem 276:7376–7382.

Kawaida R, Ohtsuka T, Okutsu J, Takahashi T, Kadono Y, Oda H, Hikita A, Nakamura K, Tanaka S, Furukawa H (2003): Jun dimerization protein 2 (JDP2), a member of the AP-1 family of transcription factor, mediates osteoclast differentiation induced by RANKL. J Exp Med 197:1029–1035.

Kenner L, Hoebertz A, Beil T, Keon N, Karreth F, Eferl R, Scheuch H, Szremska A, Amling M, Schorpp-Kistner M, Angel P, Wagner EF (2004): Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. J Cell Biol 164:613–623.

Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T (2000): Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J Exp Med 191:275–286.

Kobayashi N, Kadono Y, Naito A, Matsumoto K, Yamamoto T, Tanaka S, Inoue J (2001): Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. EMBO J 20:1271–1280.

Koide M, Maeda H, Roccisana JL, Kawanabe N, Reddy SV (2003): Cytokine regulation and the signaling mechanism of osteoclast inhibitory peptide-1 (OIP-1/hSca) to inhibit osteoclast formation. J Bone Miner Res 18: 458–465.

Kojima H, Nemoto A, Uemura T, Honma R, Ogura M, Liu Y (2001): rDrak1, a novel kinase related to apoptosis, is strongly expressed in active osteoclasts and induces apoptosis. J Biol Chem 276:19238–19243.

Komarova SV, Pilkington MF, Weidema AF, Dixon SJ,

Sims SM (2003): RANK ligand-induced elevation of cytosolic Ca2+ accelerates nuclear translocation of nuclear factor kappa B in osteoclasts. J Biol Chem 278: 8286–8293.

Korcok J, Raimundo LN, Ke HZ, Sims SM, Dixon SJ (2004): Extracellular nucleotides act through P2X7 receptors to activate NF-kappaB in osteoclasts. J Bone Miner Res 19:642–651.

Kurihara N, Menaa C, Maeda H, Haile DJ, Reddy SV (2001): Osteoclast-stimulating factor interacts with the spinal muscular atrophy gene product to stimulate osteoclast formation. J Biol Chem 276:41035–41039.

Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ, Polverino AJ (2000): Osteoprotegerin ligand modulates murine osteoclast survival *in vitro* and *in vivo*. Am J Pathol 157:435–448.

Lakkakorpi PT, Bett AJ, Lipfert L, Rodan GA, Duong le T (2003): PYK2 autophosphorylation, but not kinase activity, is necessary for adhesion-induced association with c-Src, osteoclast spreading, and bone resorption. J Biol Chem 278:11502–11512.

Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL (2000): TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488.

Laurin N, Brown JP, Morissette J, Raymond V (2002): Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. Am J Hum Genet 70:1582–1588.

Lee SE, Chung WJ, Kwak HB, Chung CH, Kwack KB, Lee ZH, Kim HH (2001): Tumor necrosis factor-alpha supports the survival of osteoclasts through the activation of Akt and ERK. J Biol Chem 276:49343–49349.

Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH, Kim HH (2002a): The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. Bone 30:71–77.

Lee SK, Kalinowski JF, Jastrzebski SL, Puddington L, Lorenzo JA (2003a): Interleukin-7 is a direct inhibitor of *in vitro* osteoclastogenesis. Endocrinology 144:3524–3531.

Lee SW, Han SI, Kim HH, Lee ZH (2002b): TAK1-dependent activation of AP-1 and c-Jun N-terminal kinase by receptor activator of NF-kappaB. J Biochem Mol Biol 35:371–376.

Lee SW, Kwak HB, Chung WJ, Cheong H, Kim HH, Lee ZH (2003b): Participation of protein kinase C beta in osteoclast differentiation and function. Bone 32:217–227.

Lee ZH, Kim HH (2003): Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. Biochem Biophys Res Commun 305:211–214.

Li X, Udagawa N, Itoh K, Suda K, Murase Y, Nishihara T, Suda T, Takahashi N (2002): p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function. Endocrinology 143:3105–3113.

Li X, Udagawa N, Takami M, Sato N, Kobayashi Y, Takahashi N (2003): p38 Mitogen-activated protein kinase is crucially involved in osteoclast differentiation but not in cytokine production, phagocytosis, or dendritic cell differentiation of bone marrow macrophages. Endocrinology 144:4999–5005.

Luchin A, Suchting S, Merson T, Rosol TJ, Hume DA, Cassady AI, Ostrowski MC (2001): Genetic and physical interactions between Microphthalmia transcription factor and PU.1 are necessary for osteoclast gene expression and differentiation. J Biol Chem 276:36703–36710.

Luegmaryr E, Glantschnig H, Wesolowski GA, Gentile MA, Fisher JE, Rodan GA, Reszka AA (2004): Osteoclast formation, survival and morphology are highly dependent on exogenous cholesterol/lipoproteins. Cell Death Differ Suppl I:S108–S118.

Mansky KC, Sankar U, Han J, Ostrowski MC (2002a): Microphthalmia transcription factor is a target of the p38 MAPK pathway in response to receptor activator of NF-kappa B ligand signaling. J Biol Chem 277: 11077–11083.

Mansky KC, Sulzbacher S, Purdom G, Nelsen L, Hume DA, Rehli M, Ostrowski MC (2002b): The microphthalmia transcription factor and the related helix-loop-helix zipper factors TFE-3 and TFE-C collaborate to activate the tartrate-resistant acid phosphatase promoter. J Leukoc Biol 71:304–310.

Matsumoto M, Sudo T, Saito T, Osada H, Tsujimoto M (2000): Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). J Biol Chem 275:31155–31161.

Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, Bachler MA, Amano H, Aburatani H, Ishikawa H, Wagner EF (2004): Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. J Biol Chem 279:26475–26480.

Matsuo K, Owens JM, Tonko M, Elliott C, Chambers TJ, Wagner EF (2000): Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. Nat Genet 24: 184–187.

Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, Pando MP, Asano T, Verma IM, Oda H, Nakamura K, Tanaka S (2000): Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. J Cell Biol 148:333–342.

Miyazaki T, Sanjay A, Neff L, Tanaka S, Horne WC, Baron R (2004): Src kinase activity is essential for osteoclast function. J Biol Chem 279:17660–17666.

Nakamura I, Rodan GA, Duong LT (2003): Distinct roles of p130Cas and c-Cbl in adhesion-induced or macrophage colony-stimulating factor-mediated signaling pathways in prefusion osteoclasts. Endocrinology 144: 4739–4741.

Nilsson SK, Lieschke GJ, Garcia-Wijnen CC, Williams B, Tzelepis D, Hodgson G, Grail D, Dunn AR, Bertoncello I (1995): Granulocyte-macrophage colony-stimulating factor is not responsible for the correction of hematopoietic deficiencies in the maturing op/op mouse. Blood 86:66–72.

Novack DV, Yin L, Hagen-Stapleton A, Schreiber RD, Goeddel DV, Ross FP, Teitelbaum SL (2003): The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis. J Exp Med 198:771–781.

Oba Y, Chung HY, Choi SJ, Roodman GD (2003): Eosinophil chemotactic factor-L (ECF-L): a novel osteoclast stimulating factor. J Bone Miner Res 18: 1332–1341.

Oursler MJ (1994): Osteoclast synthesis and secretion and activation of latent transforming growth factor beta. J Bone Miner Res 9:443–452.

Owens JM, Matsuo K, Nicholson GC, Wagner EF, Chambers TJ (1999): Fra-1 potentiates osteoclastic differentiation in osteoclast-macrophage precursor cell lines. J Cell Physiol 179:170–178.

Reddy SV, Takahashi S, Dallas M, Williams RE, Neckers L, Roodman GD (1994): Interleukin-6 antisense deoxyoligonucleotides inhibit bone resorption by giant cells from human giant cell tumors of bone. J Bone Miner Res 9:753–757.

Reddy SV, Roodman GD (1998): Control of osteoclast differentiation. Crit Rev Eukaryot Gene Expr 8:1–17.

Roodman GD (1999): Cell biology of the osteoclast. Exp Hematol 27:1229–1241.

Sanjay A, Houghton A, Neff L, DiDomenico E, Bardelay C, Antoine E, Levy J, Gailit J, Bowtell D, Horne WC, Baron R (2001): Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. J Cell Biol 152:181–195.

Sato T, Shibata T, Ikeda K, Watanabe K (2001): Generation of bone-resorbing osteoclasts from B220+ cells: Its role in accelerated osteoclastogenesis due to estrogen deficiency. J Bone Miner Res 16:2215–2221.

Schwartzberg PL, Xing L, Hoffmann O, Lowell CA, Garrett L, Boyce BF, Varmus HE (1997): Rescue of osteoclast function by transgenic expression of kinase-deficient Src in src-/- mutant mice. Genes Dev 11:2835–2844.

Shin JN, Kim I, Lee JS, Koh GY, Lee ZH, Kim HH (2002): A novel zinc finger protein that inhibits osteoclastogenesis and the function of tumor necrosis factor receptor-associated factor 6. J Biol Chem 277:8346–8353.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ, et al. (1997): Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89:309–319.

Soriano P, Montgomery C, Geske R, Bradley A (1991): Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64:693–702.

Steinbeck MJ, Appel WH Jr, Verhoeven AJ, Karnovsky MJ (1994): NADPH-oxidase expression and *in situ* production of superoxide by osteoclasts actively resorbing bone. J Cell Biol 126:765–772.

Sugatani T, Alvarez U, Hruska KA (2003): PTEN regulates RANKL- and osteopontin-stimulated signal transduction during osteoclast differentiation and cell motility. J Biol Chem 278:5001–5008.

Takahashi S, Goldring S, Katz M, Hilsenbeck S, Williams R, Roodman GD (1995): Downregulation of calcitonin receptor mRNA expression by calcitonin during human osteoclast-like cell differentiation. J Clin Invest 95:167–171.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T (2002b): Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3:889–901.

Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, Yokochi T, Oda H, Nakamura K, Ida N, Wagner EF, Taniguchi T (2002a): RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature 416:744–749.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T (2000): T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature 408: 600–605.

Takeshita S, Namba N, Zhao JJ, Jiang Y, Genant HK, Silva MJ, Brodt MD, Helgason CD, Kalesnikoff J, Rauh MJ, Humphries RK, Krystal G, Teitelbaum SL, Ross FP (2002): SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. Nat Med 8:943–949.

Takuma A, Kaneda T, Sato T, Ninomiya S, Kumegawa M, Hakeda Y (2003): Dexamethasome enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts. J Biol Chem 278:44667–44674.

Tanaka S, Amling M, Neff L, Peyman A, Uhlmann E, Levy JB, Baron R (1996): c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature 383:528–531.

Teitelbaum SL, Ross FP (2003): Genetic regulation of osteoclast development and function. Nat Rev Genet 4:638–649.

Theoleyre S, Wittrant Y, Couillaud S, Vusio P, Berreur M, Dunstan C, Blanchard F, Redini F, Heymann D (2004): Cellular activity and signaling induced by osteoprotegerin in osteoclasts: Involvement of receptor activator of nuclear factor ~~k~~B ligand and MAPK. Biochem Biophys Acta 1644:1–7.

Tondravi MM, McKercher SR, Anderson K, Erdmann JM, Quiroz M, Maki R, Teitelbaum SL (1997): Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. Nature 386:81–84.

Udagawa N, Chan J, Wada S, Findlay DM, Hamilton JA, Martin TJ (1996): c-fos antisense DNA inhibits proliferation of osteoclast progenitors in osteoclast development but not macrophage differentiation *in vitro*. Bone 18:511–516.

Udagawa N, Takahashi N, Katagiri T, Tamura T, Wada S,

Findlay DM, Martin TJ, Hirota H, Taga T, Kishimoto T, et al. (1995): Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. J Exp Med 182:1461–1468.

Vignery A (2000): Osteoclasts and giant cells: Macrophage-macrophage fusion mechanism. Int J Exp Pathol 81:291–304.

Wagner EF, Matsuo K (2003): Signaling in osteoclasts and the role of Fos/AP1 proteins. Ann Rheum Dis 62:83–85.

Weilbaecher KN, Motyckova G, Huber WE, Takemoto CM, Hemesath TJ, Xu Y, Hershey CL, Dowland NR, Wells AG, Fisher DE (2001): Linkage of M-CSF signaling to Mitf, TFE3, and the osteoclast defect in Mitf (mi/mi) mice. Mol Cell 8:749–758.

Wong BR, Besser D, Kim N, Arron JR, Vologodskaia M, Hanafusa H, Choi Y (1999): TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol Cell 4: 1041–1049.

Wu X, McKenna MA, Feng X, Nagy TR, McDonald JM (2003): Osteoclast apoptosis: The role of Fas *in vivo* and *in vitro*. Endocrinology 144:5545–5555.

Xing L, Bushnell TP, Carlson L, Tai Z, Tondravi M, Siebenlist U, Young F, Boyce BF (2002): NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. J Bone Miner Res 17:1200–1210.

Xing L, Venegas AM, Chen A, Garrett-Beal L, Boyce BF, Varmus HE, Schwartzberg PL (2001): Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. Genes Dev 15:241–253.

Yan T, Riggs BL, Boyle WJ, Khosla S (2001): Regulation of osteoclastogenesis and RANK expression by TGF-beta1. J Cell Biochem 83:320–325.

Yang S, Madyastha P, Bingel S, Ries W, Key L (2001): A new superoxide-generating oxidase in murine osteoclasts. J Biol Chem 276:5452–5458.

Yang CR, Wang JH, Hsieh SL, Wang SM, Hsu TL, Lin WW (2004): Decoy receptor 3 (DcR3) induces osteoclast formation from monocyte/macrophage lineage precursor cells. Cell Death Differ Suppl I:S97–S107.

Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD (1990): The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442–444.

Zaidi M, Moonga BS, Huang CL (2004): Calcium sensing and cell signaling processes in the local regulation of osteoclastic bone resorption. Biol Rev Camb Philos Soc 79:79–100.

Zhang YH, Heulsmann A, Tondravi MM, Mukherjee A, Abu-Amer Y (2001): Tumor necrosis factor-alpha (TNF) stimulates RANKL induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. J Biol Chem 276:563–568.

Zhang Z, Jimi E, Bothwell AL (2003): Receptor activator of NF-kappaB ligand stimulates recruitment of SHP-1 to the complex containing TNFR-associated factor 6 that regulates osteoclastogenesis. J Immunol 171:3620–3626.